

# T-cadherin suppresses angiogenesis in vivo by inhibiting migration of endothelial cells

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**Abstract** Our previous studies have revealed the abundant expression of T-cadherin—a glycosylphosphatidylinositol (GPI)-anchored member of cadherin superfamily—in endothelial and mural cells in the heart and vasculature. The upregulation of T-cadherin in vascular proliferative disorders such as atherosclerosis and restenosis suggests the involvement of T-cadherin in vascular growth and remodeling. However, the functional significance of this molecule in the vasculature remains unknown. The effect of T-cadherin on angiogenesis in vivo was evaluated using Matrigel implant model. We demonstrate that T-cadherin overexpression in L929 cells injected in Matrigel inhibits neovascularization of the plug. In vitro T-cadherin inhibits the directional migration of endothelial cells, capillary growth, and tube formation but has no effect

on endothelial cell proliferation, adhesion, or apoptosis in vitro. These data suggest that T-cadherin expressed in the stroma could act as a negative guidance cue for the ingrowing blood vessels and thus could have an important potential therapeutic application.

**Keywords** Angiogenesis · Blood vessels · Cadherin · Endothelial cells · Navigating receptor · Vascular biology

## Introduction

Excessive or insufficient angiogenesis in adult tissues contributes to many pathologies, including atherosclerosis and tumor growth. The migratory behavior of endothelial cells and their ability to form a new vascular tree plays a key role in this process. Cadherins are known to be crucial for vessel growth and maturation [1, 2]. Cadherins comprise a large superfamily of molecules responsible for Ca<sup>+</sup>-dependent cell recognition and adhesion. Classical cadherins consist of five extracellular (EC1-EC5), as well as transmembrane and cytoplasmic domains [2, 3]. During embryogenesis cadherins mediate cell sorting and segregation, cell migration, morphogenesis, axon growth, and target pathfinding. In adult organisms cadherins regulate the stable organization of solid tissues, the orderly turnover of cells in rapidly growing tissues, the physiological barrier function of bordering tissues, and selective transport of solutions and cells [3, 4]. The predominantly expressed cadherins in vascular cells, such as endothelial, pericytes and vascular smooth muscle cells (VSMC) are N-cadherin/R-cadherin and T-cadherin [1, 2, 5].

T-cadherin is an atypical member of the cadherin superfamily. While possessing the general extracellular structure of classical cadherins, T-cadherin lacks transmembrane

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and cytoplasmic domains and is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety [6]. Since transmembrane and cytoplasmic domains of classical cadherins are generally recognized to be crucial in maintaining stable cell–cell contacts [3], it is likely that T-cadherin is implicated in cell signaling rather than cell adhesion [5, 7, 8].

The upregulation of T-cadherin expression in vascular cells in adventitial *vasa vasorum* of injured arteries, in vascular proliferative disorders such as atherosclerosis and restenosis, as well as in tumor associated neovascularization, suggests the involvement of T-cadherin in pathological angiogenesis [9–12]. However, the role of T-cadherin in the vasculature is poorly understood.

In our lab, T-cadherin has been isolated as a receptor responsible for hormone-like signaling effects of low density lipoproteins (LDL) [13] and was shown to mediate LDL-induced cell signaling and cell migration in vitro [14]. Adenoviral overexpression of T-cadherin in HUVEC (human umbilical endothelial cells), facilitates spontaneous cell migration, and change of the phenotype from quiescent to promigratory [15]. It is likely that homophilic interaction of T-cadherin in endothelial cells results in activation of small G-proteins, RhoA/ROCK, and Rac pathways with subsequent reorganization of actin cytoskeleton and stress fiber formation [15]. However, in vivo effects of T-cadherin for angiogenesis remain to be investigated.

The purpose of this study was to define the role of T-cadherin in angiogenesis in vivo and to elucidate the possible mechanisms of T-cadherin functioning in the vascular system. We demonstrate that T-cadherin could act as a negative guidance cue for growing vessels. Using Matrigel implant we show that T-cadherin expression in the stroma inhibits neoangiogenesis. T-cadherin expressed on the cells surface mediates homophilic interaction and contact inhibition resulting in suppressed endothelial cell migration.

## Materials and methods

### Cell culture and transfection

As previously described [14, 16], L929 cells were stably transfected with T-cadherin (pcDNA 3.1) using Lipofectamin 2000 reagent. Luciferase cDNA fragment in antisense orientation was cloned into the pcDNA 3.1 vector and used as a control. T-cadherin expression in control and T-cadherin transfectants was examined using Western blotting [14]. For Matrigel experiments, L929 cell were maintained in DMEM containing 100 U/ml penicillin, 100 U/ml streptomycin, and 10% fetal bovine serum (DMEM/FBS) at 37°C and 5% CO<sub>2</sub>.

### Matrigel implantation into NUDE mice

All animal work was approved by Institutional Ethical Committee (Cardiology Research Complex). 400 µl of ice-cold Matrigel matrix (BD) was premixed with  $1.7 \times 10^6$  L939 cells (either expressing T-cadherin or control plasmid) in 100 µl of complete DMEM. NUDE mice of 8–12 weeks were anesthetized with by i.p. injection of 2.5% avertin. 500 µl of cell suspension in Matrigel was injected subcutaneously into the right and left sides of the mouse back (spatula region) using 22G needle. Around 3, 7, 10, and 14 days after injection mice were sacrificed and the entire Matrigel plugs were isolated. Quantification of blood vessels was achieved using immunofluorescent visualization of blood vessels on frozen Matrigel sections and by measuring the amount of hemoglobin contained in the plugs [17]. Weight measuring of the Matrigel plugs was followed by dividing each plug into two parts: one was immediately used for hemoglobin assessment and the other was placed in O.C.T. Compound (Sakura Inc., Tokyo, Japan) and frozen in liquid nitrogen for immunofluorescent analysis.

### Hemoglobin assessment

Hemoglobin content was analyzed using cyanhemoglobin method (Drabkin's method) modified by us for tissues. Briefly, fragments of Matrigel plugs were weighed and homogenized in 200 µl of 0.9% NaCl saline using electric homogenizer (Janke & Kunkel, IKA-Labor Technik) following by centrifugation at 5,000 rpm for 7 min. Supernatant was cleared by adding 100 µl of chloroform per 50 µg of Matrigel specimen with subsequent centrifugation at 13,000 rpm for 3 min. Hemoglobin in the specimen was converted to CN-derivative by adding 150 µl of Drabkin's solution containing K<sub>3</sub>Fe(CN)<sub>6</sub>, KCN, KH<sub>2</sub>PO<sub>4</sub>, and TritonX-100; the sample was incubated at room temperature for 15 min and A<sub>594</sub> absorbance was measured using spectrophotometer (Janako).

### Immunofluorescent staining of blood vessels

Blood vessels were visualized by immunofluorescent staining of 6 µm frozen Matrigel sections using rat anti-mouse CD31 antibody (BD Pharmingen<sup>TM</sup>, 0.125 µg/ml in BSA/normal serum solution) as described previously [17, 18]. This was followed by incubation with Alexa 594-conjugated anti-rat antibody (Molecular Probes, 10 µg/ml in PBS). To assess the number of mature vessels, we performed double immunofluorescent staining with rat anti-mouse CD31 antibody combined with rabbit anti-mouse α-actin (Epitomics, 1:500) or rabbit anti-NG2 (Chemicon, 1:200) antibodies to visualize smooth muscle cell (SMCs)

or pericytes, respectively. This was followed by incubation with the mixture of secondary antibody: Alexa 594-conjugated anti-rat antibody and Alexa 488-conjugated donkey anti-rabbit antibody. Cell nuclei were counterstained with DAPI (Molecular Probes). For negative controls rat or rabbit non-specific IgGs were used in appropriate concentration. Images were obtained using Zeiss Axiovert 200M microscope equipped with CCD camera AxioCam HRc and Axiovision software (Zeiss) and further processed using Adobe PhotoShop software (Adobe Systems).

#### Quantification of vessel density (VD) and statistical analysis

Vessel size and density was evaluated using MetaMorph 5.0 (Universal Imaging) and Adobe PhotoShop software (Adobe Systems). VD was determined as the number of CD31-positive vessels in a field normalized to the DAPI-stained area unit of each Matrigel section. For the quantification of VD, vessel numbers were counted in 4–5 fields of view ( $1.107 \text{ mm}^2$ ) on three random sections for each Matrigel plug at  $100\times$  total magnification ( $10\times$  objective). All vessels were separated into three groups: capillaries (CD31-positive vessels without lumen and with length less than  $20 \mu\text{m}$ ); medium vessels (CD31-positive vessels with length  $20\text{--}40 \mu\text{m}$ ) and large vessels (with diameter  $>40 \mu\text{m}$ ); the number of vessels in each group was counted separately. All experiments were repeated at least three different times in triplicates. Data are expressed as mean  $\pm$  SEM. Data were analyzed using Mann–Whitney U-criteria. Differences with  $P < 0.05$  were considered as statistically significant.

#### HUVEC proliferation

Effect of T-cadherin on proliferation of HUVEC was analyzed either by co-cultivating HUVEC with T-cadherin-positive or T-cadherin-negative cells, or by cultivating HUVEC using conditioned medium from T-cadherin-negative or T-cadherin-positive cells. The conditioned medium from L939 was centrifuged at  $1,000g$  for 10 min, concentrated 100 times using Centricon YM-10, Millipore filter, and supplemented with 1–10% of FBS and  $200 \mu\text{g/ml}$  ECGF (RELIA Tech GmbH, Germany). For co-cultivating assay, HEK293 cells expressing GFP/T-cadherin or GFP/control plasmid [14] and HUVEC were seeded in co-culture on fibronectin (Sigma) coated cover glass in the ratio of 1:5, 1:2, and 1:1, and incubated in at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  for 48 h. Cells were fixed in 4% paraformaldehyde and ethanol and processed for immunofluorescent staining as described earlier. Human PCNA antigen (proliferating cell nuclear antigen) (DakoCytoma-

tion) was used as a marker of proliferating cells; this was followed by incubation with Alexa-594 conjugated secondary antibody. Proliferating HUVEC cells showed red fluorescence, while proliferating HEK293/GFP cells had double fluorescence in the green and red channels. The number of PCNA-positive HUVEC was calculated in 4–5 fields of view on each cover glass at  $200\times$  total magnification; the obtained number of PCNA-positive HUVEC cells was normalized to the total number of HUVEC for each field of view. Data were analyzed using Mann–Whitney U-criteria.

#### Generation of T-cadherin fragments

As described earlier, human cDNA encoding EC1 and EC5 domains of T-cadherin were obtained by PCR [19], cloned into pET-21 a(+) (Novagen) vector, and overexpressed in *Escherichia coli* strain BL 21 (DE3) by induction with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) for 4 h. Using the procedures described by the manufacturers, the proteins were purified on a nickel affinity column (Ni-NTA agarose, Qiagen GmbH, Hilden, Germany) from the supernatant of lysed cells. T-cadherin domains were eluted from the column with 250 mM imidazole, dialyzed against PBS, and sterilized by passing through  $0.22\text{-}\mu\text{m}$  filter. Protein concentration was determined by the Bradford assay with BSA (Sigma) as a standard (Bio-Rad).

#### HUVEC adhesion

The effect of T-cadherin on HUVEC adhesion was examined using either T-cadherin domains precoated plastic dishes or a monolayer of T-cadherin-positive or T-cadherin-negative cells. In the first case, adhesion assays were performed as described earlier with some modifications [19]. Briefly, 24-well dishes were precoated overnight at  $4^\circ\text{C}$  with 0.1% gelatin including EC1 or EC5 (control) recombinant domains of T-cadherin in concentration  $0.1 \text{ mg/ml}$  or BSA. After absorption the dishes were washed with sterile PBS. Prior to adhesion assay, HUVEC were incubated for 12 h at  $37^\circ\text{C}$  in a solution of  $0.2 \mu\text{g/ml}$  fluorescently labeled acetylated low-density lipoprotein (Ac-LDL-Alexa488 (Invitrogen)) as described [20]. Specifically labeled HUVEC were seeded on the precoated plates at a density  $8 \times 10^4$  cells/well and cultured (DMEM supplemented by 10% FBS (HyClone),  $200 \mu\text{g/ml}$  ECGF (RELIA Tech GmbH, Germany), 5 U/ml heparin, 1 mM sodium pyruvate (GIBCO BRL), 20 mM HEPES (Helicon),  $100 \mu\text{g/ml}$  penicillin, and  $100 \mu\text{g/ml}$  streptomycin (GIBCO BRL)). In the second case, L929 T-cadherin-positive or T-cadherin-negative cells were seeded at various densities ( $2 \times 10^4\text{--}10^5$  cells/well) on 24-well dishes and

cultivated until 50–100% cell monolayer. HUVEC pre-labeled with Ac-LDL-Alexa488 were seeded on top of L929 T-cadherin-positive or T-cadherin-negative cells. Cells in each case were incubated at 37°C for 2 h and examined by fluorescent Axiovert 200M microscope equipped with CO<sub>2</sub> incubator and heating stage (Zeiss). Live images were taken using CCD camera AxioCam HRc (Zeiss) at 200× total magnification in 4–5 fields of view for each well and analyzed with Axiovision software (Zeiss). All experiments were repeated at least three times in triplicates. Data are expressed as mean ± SEM. Data were analyzed using Mann–Whitney U-criteria. Differences with  $P < 0.05$  was considered as statistically significant.

#### Apoptosis assay

The effect of recombinant fragments of T-cadherin on endothelial cells apoptosis was assessed using Caspase-3 Colorimetric Assay (R&D Systems, CN BF3100) according to manufacturer's instructions. Briefly, culture dishes were pre-coated for 12 h at +4°C with 0.2% gelatin containing either 0.1 mg/ml of EC1 or EC5 domain or 0.1 mg/ml of BSA. HUVEC were detached by trypsinisation and seeded onto pre-coated substrate at a density of  $7 \times 10^4$  HUVEC per cm<sup>2</sup> in the endothelium growth medium. After incubation for 18 h at 37°C, cells were detached from the plastic dish using 0.02% EDTA solution and centrifuged at 1,000g for 10 min. Cell pellet was lysed by addition of the Lysis buffer supplied with the kit. The cell lysates were added to 96 well microplate and incubated with caspase-3 substrate at 37°C for 2 h. Absorbance in wells was measured at 405 nm. As a positive control HUVECs were incubated for 18 h in the growth medium containing 1% FBS and 1nM staurosporine (Sigma) to induce apoptosis in endothelial cells. All experiments were repeated three times in triplicates. Data were expressed as mean ± SEM. Data were analyzed using Mann–Whitney U-criteria. Differences with  $P < 0.05$  was considered as statistically significant.

#### Tube formation assay

The formation of vascular-like structures by HUVECs on growth factor-reduced Matrigel (BD Biosciences) was performed as previously described [21] according to the manufacturer's instructions. Around  $5 \times 10^4$  of HUVEC (passage 2–4) were seeded on Matrigel and incubated at 37°C for 24 h in the complete endothelial cells growth medium.

To evaluate the effect of immobilized T-cadherin on tube formation we polymerized Matrigel and then allowed EC1 or EC5 (control) peptide solutions (0.1 mg/ml) or PBS

to infiltrate Matrigel overnight. For positive control HUVECs were allowed to form tubes in the complete endothelial cells growth medium, for negative control tubes were formed in the growth factor-reduced medium (0.5% FBS without ECGF). To assess the effect of soluble EC1 or EC5 domains, cells were incubated on polymerized Matrigel in the presence of 0.1 mg/ml soluble recombinant peptides. The average length of formed capillary-like structures was quantified by measuring the length of tubes in five randomly chosen fields from each well, using MetaMorph 5.0 (Universal Imaging) software. All experiments were repeated three times in triplicates. Data are expressed as mean ± SEM. Data were analyzed using Mann–Whitney U-criteria. Differences with  $P < 0.05$  were considered as statistically significant.

#### Rat ring aorta assay

To study the action of T-cadherin on angiogenesis we used cultured aorta explants in three-dimensional matrix gels [22] with some modifications. Around 8–12-week-old Wistar male rats were sacrificed and thoracic aortas were immediately placed into a cold serum-free essential medium (RPMI1640, GIBCO). The peri-aortic fibroadipose tissue was carefully removed and 3 mm-long aortic rings (approximately 8 per aorta) were sectioned and embedded in growth factors reduced Matrigel (BD Pharmingen), containing EC1 or EC5 (control) T-cadherin domains (at various concentrations: 0.0004; 0.001; 0.002; 0.02 mg/ml) or PBS. Matrigels were kept at 37°C in a humidified incubator for 14 days and examined every second day. Experiments were repeated 4 times in triplicates. The area covered by sprouted capillaries per 1 mm of ring side, as well as average length of sprouts were analyzed using MetaMorph 5.0 (Universal Imaging) software. All experiments were repeated three times in triplicates. Data were expressed as mean ± SEM. Data were analyzed using Mann–Whitney U-criteria. Differences with  $P < 0.001$  was considered as statistically significant.

#### HUVEC migration in Boyden chamber

Migration of HUVEC was assessed in Boyden chamber using 8 μm pore size membrane (NeuroProbe Inc.) as described previously [23]. Briefly,  $1 \times 10^5$  serum-deprived HUVEC were suspended with 0.05% trypsin-EDTA (Invitrogen) and seeded in serum-free DMEM on top of the membrane in Boyden chamber. HUVEC were allowed to migrate for 4 hrs at 37°C using 1–10% of FBS as chemoattractant. Non-migrated cells were removed; membrane with cells was fixed in 100% ethanol and

stained in Diff-Quick, scanned, and analyzed using Image-J software (National Institute of Health).

To assess the contact-dependent effect of T-cadherin on HUVEC migration, the membrane on its upper side was precoated with 0.2% gelatin solution containing 0.1 mg/ml of recombinant EC1 or EC5 (control) domains. After incubation for 12 h at 37°C the membrane was analyzed in HUVEC migration assay. HUVEC were allowed to migrate to the growth medium containing 1.5 or 10% of FBS. To analyze the effect of soluble domains, 0.1 mg/ml of peptides was added to the lower chamber.

To examine the possibility of T-cadherin shedding and the effect of the soluble T-cadherin on HUVEC migration, as a chemoattractant we used conditioned medium from T-cadherin-negative or T-cadherin-positive L939 cells in the presence of 1–10% of FBS and appropriate supplements. The conditioned medium was centrifuged at 1,000g for 10 min and concentrated 100 times using Centricon YM-10, Millipore filter. All experiments were repeated three times in triplicates. Data are expressed as mean  $\pm$  SEM. Data were analyzed using Mann–Whitney U-criteria. Differences with  $P < 0.05$  were considered as statistically significant.

## Results

### Local T-cadherin overexpression in Matrigel plugs

To reveal the role of T-cadherin in the growth of blood vessels in an *in vivo* model, we have subcutaneously injected Matrigels containing T-cadherin-positive or T-cadherin-negative L939 cells into the spatula region of the mouse back. The cells implanted in Matrigel retained their viability up to 14 days, assuring the sustained expression of T-cadherin over the course of the entire experiment (our unpublished observations). Matrigel plugs were isolated 3, 7, 10, and 14 days after injection, and their vascularization by vessels outgrowing from *a. subclavia* was assessed. In all cases Matrigel implants with L939 cells overexpressing T-cadherin were smaller in size, had a yellowish color, and newly-formed vessels were smaller in diameter (Fig. 1A).

At the day 14, plugs with T-cadherin expressing cells were significantly smaller in weight ( $0.28 \pm 0.02$  vs.  $0.45 \pm 0.02$  in control,  $P < 0.01$ ), which was accounted for by the clearly pronounced difference in vascularization. The hemoglobin content normalized to the weight of the analyzed fragment of Matrigel was significantly lower in T-cadherin plugs ( $0.74 \pm 0.04$  vs.  $1.17 \pm 0.08$  in control,  $P < 0.05$ ). The difference in hemoglobin content, reflecting the blood flow, becomes evident at the day 10, the time normally required for the Matrigel plug to develop a

vascular network [17, 24]. Dynamic analysis over the whole period of incubation (from day 3–day 14) demonstrated that the hemoglobin content in Matrigel plugs overexpressing T-cadherin was lower at all tested time points (Fig. 1B). Thus, injection of cells overexpressing T-cadherin suppresses vessel ingrowth into the Matrigel implant.

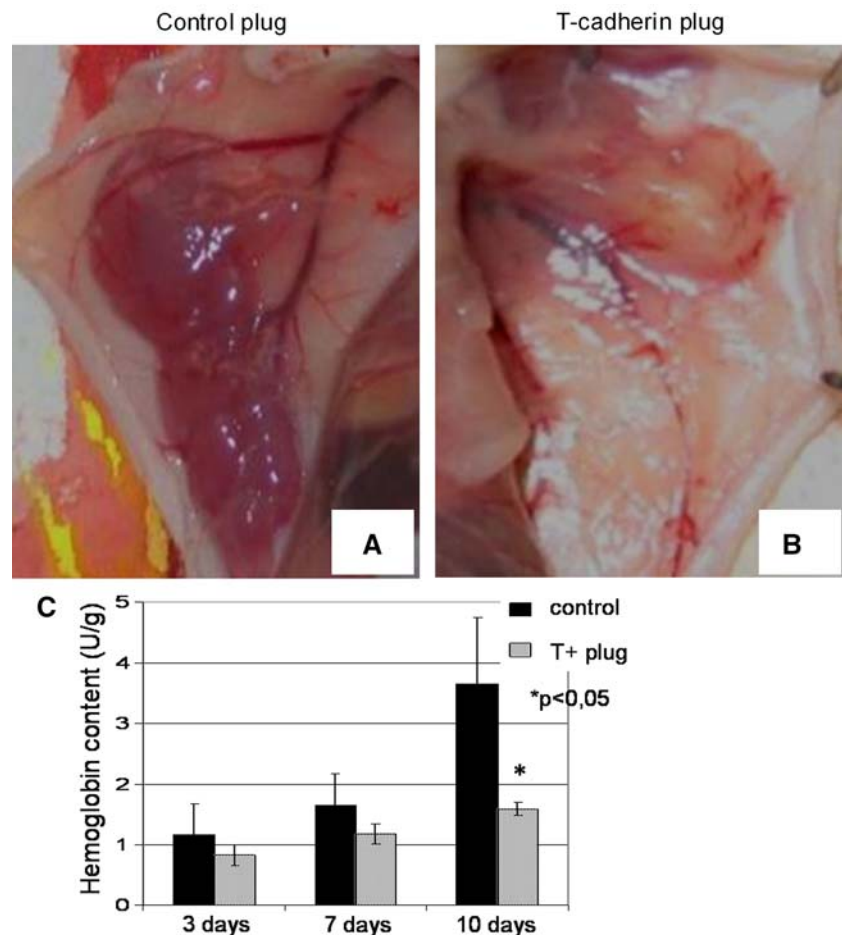
### T-cadherin overexpression suppresses capillaries but not mature vessels

To clarify which stage of vessel growth or maturation is affected by T-cadherin expressed in the surrounding tissue, we have analyzed the vessel density, size, and the type/quality of blood vessels. Matrigel sections were taken from various parts of the plug and stained with antibody against endothelial marker CD31 (Fig. 2A, B). There was no difference in the vascular density for large vessels (with lumen diameter  $>40 \mu\text{m}$ ) between control and T-cadherin plugs ( $1.25 \pm 0.22$  vs.  $1.27 \pm 0.33$ ;  $P > 0.05$ ), whereas for small vessels (with lumen diameter  $<40 \mu\text{m}$  and length  $>20 \mu\text{m}$ ) the vessel density was significantly lower in the plugs overexpressing T-cadherin ( $91.5 \pm 7.2$  vs.  $207.6 \pm 9.7$ ;  $P < 0.05$ ). The density of capillaries (vessels without visible lumen or with length  $<20 \mu\text{m}$ ) was also significantly lower in the plugs overexpressing T-cadherin compared to control (Fig. 2E). In Matrigel plugs with control L929 cell vast majority of vessels expressed T-cadherin. However, in Matrigel plugs with T-cadherin expressing L929 cells, the number of T-cadherin-positive vessels was significantly lower (Fig. 2C, D, F).

One of the possible reasons for the reduced vessel density of capillaries and small vessels in the plugs with T-cadherin-positive cells could be an impaired stabilization of the newly-forming vessels. To find out if local expression of T-cadherin by L929 fibroblasts in the Matrigel affects maturation of blood vessels we evaluated the density of vessels surrounded by mural cells. Mature vessels were revealed by double immunostaining with antibodies against endothelial (CD31) combined with antibody against  $\alpha$ -SM actin (SMCs) or antibody against pericytes (NG2 antigen) [25, 26]. There was no difference in vessel density of double positive CD31/ $\alpha$ -SM actin or CD31/NG2 vascular structures (Fig. 3), indicating that T-cadherin overexpression exerts no effect on vessel maturation in Matrigel implants.

Cadherin recombinant fragments are used extensively for *in vitro* evaluation of cadherin effects on cell adhesion, proliferation, and cell sorting [19, 27]. To reveal the role of T-cadherin in endothelial cell adhesion, proliferation, apoptosis, and migration we used its recombinant fragments in our *in vitro* experiments.

**Fig. 1** Matrigel plugs. **(A)** Macroscopic view of representative Matrigel plugs containing L939 cells: T-cadherin-negative cells (control) or T-cadherin positive cells. **(B)** Hemoglobin content in Matrigel implants measured by cyanhemoglobin method at 3, 7, and 10 days. Data presented as mean  $\pm$  SEM from 14 plugs; \* $P < 0.05$



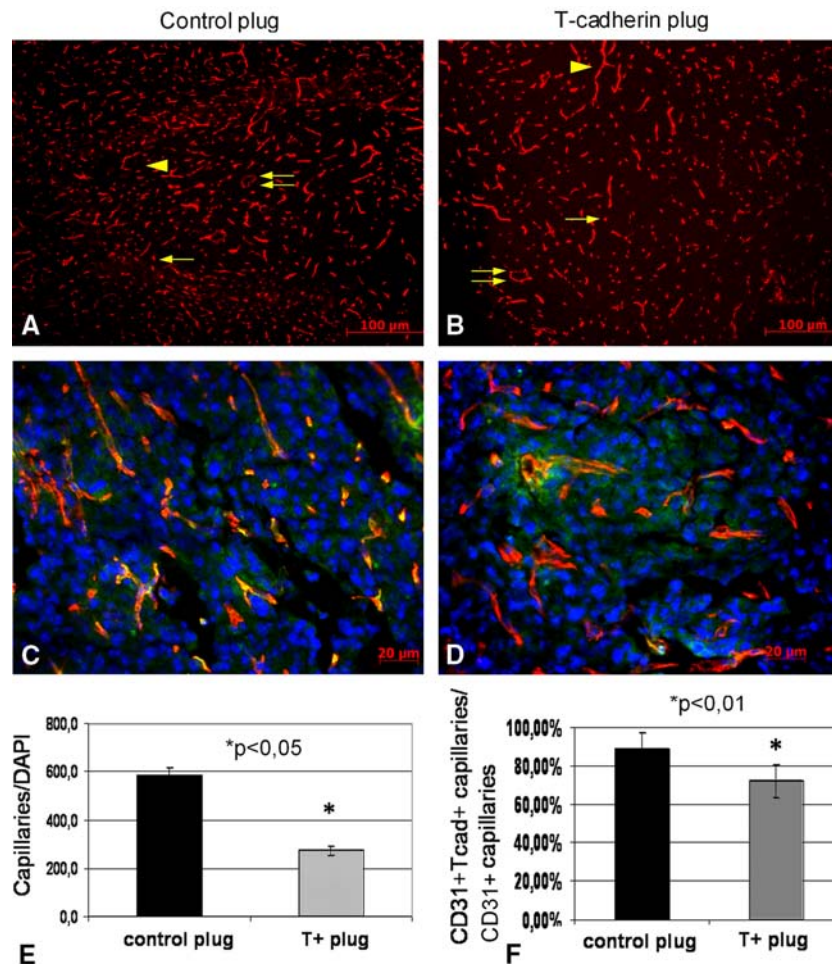
### Recombinant N-terminal domain of T-cadherin inhibits angiogenesis in vitro

To confirm that T-cadherin acts as angiogenesis inhibitor we tested the ability of recombinant T-cadherin fragments to inhibit the formation and growth of capillary network in the Matrigel tube and rat ring aorta assays in vitro. Amino-terminal EC1 and juxtamembrane EC5 extracellular domains of T-cadherin were aseptically purified in LPS-free system. We have previously characterized and used recombinant T-cadherin and its domains in a number of studies to mimic T-cadherin action [19]. EC1 domain of cadherins is known to be crucial for cell recognition and formation of cell–cell contacts, while EC5 domain is frequently used as a negative control [27]. Incubation of HUVEC, which endogenously expresses T-cadherin, in the presence of soluble EC1 or EC5 domains in 0.1 mg/ml concentration in the media did not affect formation of capillary-like structures in the capillary tube assay (Fig. 4). However, immobilization of EC1, but not of EC5 domain, on Matrigel suppressed tubulogenesis (Fig. 4). Consistent with this, in the rat aortic ring assay the immobilization of

EC1 on Matrigel resulted in a marked reduction (2–3-fold) of capillary growth and sprouting compared to EC5 domain. The effect of EC1 was evident at all time points (from 3 days to 14 days) and was dose-dependent (Fig. 5). These data indicate that in contrast to soluble T-cadherin fragments, immobilized EC1 domain of T-cadherin, which mimics homophilic cell–cell interactions [25], significantly suppresses angiogenesis in in vitro models.

T-cadherin specifically targets endothelial cell migration, but not proliferation, apoptosis or cell sorting

To further investigate the mechanism of the inhibitory effect of T-cadherin on angiogenesis we examined its action on endothelial cell proliferation, apoptosis, and migration. Co-culturing of either L939 or HEK293 (T-cadherin-positive or control cells) with HUVEC exerted no effect on the number of PCNA-positive endothelial cells (Fig. 6A). There was also no inhibitory effect of T-cadherin on HUVEC adhesion, since no significant difference in the number of adherent HUVEC seeded on EC1 or EC5



**Fig. 2** Vascularization of Matrigel plugs. (**A, B**) Immunofluorescent staining of frozen Matrigel section with antibodies against endothelial marker CD31 (red fluorescence). Large vessels are indicated by the arrowheads, small vessels—by double arrows and capillaries—by single arrows. Scale bars—100  $\mu$ m. (**C, D**) Double immunofluorescent staining of T-cadherin (green fluorescence) and CD31 (red fluorescence) on sections of control Matrigel plug (**C**) and Matrigel with T-cadherin expressing cells (**D**). Yellow staining represents blood vessels expressing T-cadherin, while red staining—vessels

which do not express T-cadherin. Nuclei are counterstained with DAPI. (**E**) The density of capillaries was determined as the number of CD31-positive vessels in a field normalized to the DAPI-stained area unit of each Matrigel section. (**F**) The proportion of capillaries expressing T-cadherin was determined as the number of double stained vessels (CD31/T-cadherin) to the total number of vessels (CD31). Data present as mean  $\pm$  SEM of 42 sections; \* $P < 0.05$ . Scale bars—20  $\mu$ m

domains or BSA precoated plastic was registered. Similarly, T-cadherin expression on the cell surface did not interfere with HUVEC adhesion onto a monolayer of L929 cells (data not shown). Furthermore, seeding HUVEC on plastic coated with gelatin including EC1 domain (0.1 mg/ml) did not cause the activation of caspase-3, a key switch of the apoptosis in HUVEC (Fig. 6B). These data suggest that the inhibitory effect of T-cadherin is not related to the down-regulation of endothelial cell proliferation or adhesion or apoptosis induction.

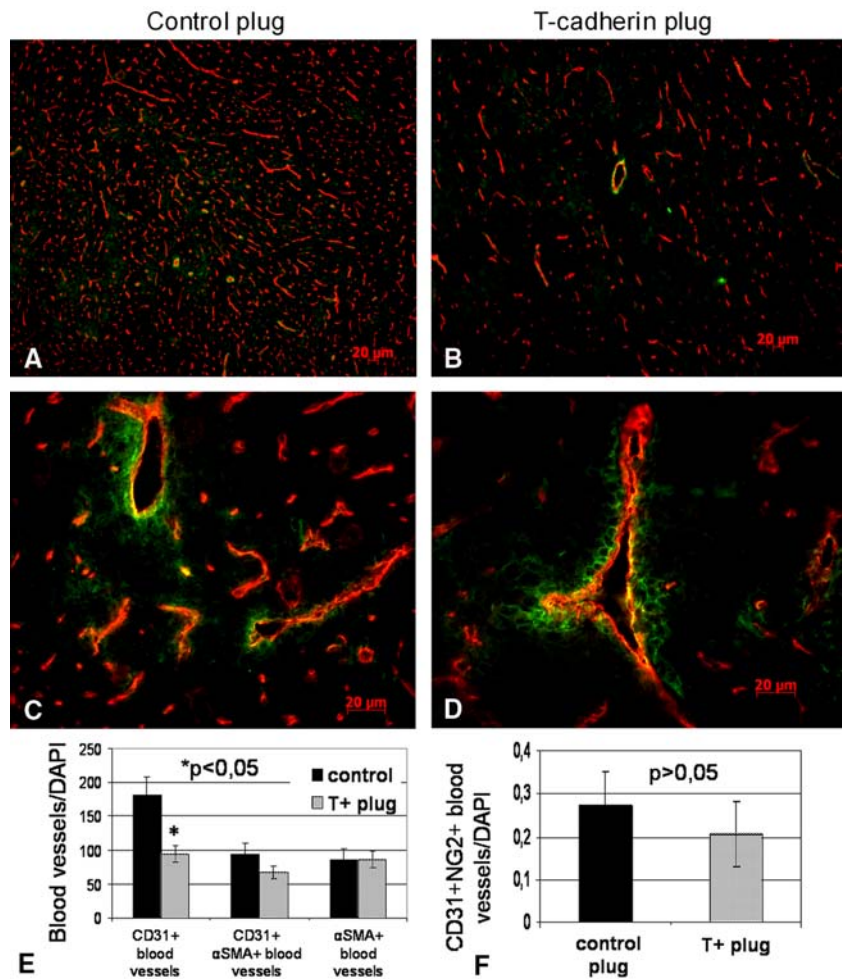
However, the migration of HUVEC in Boyden chamber through the membrane coated with EC1 domain, important for cell–cell interaction, was reduced by 2-folds compared to control (EC5) domain (Fig. 6C), indicating that T-cadherin could act as an inhibitor of endothelial cell

migration. This assumption is also supported by the results of the capillary tube (Fig. 4) and rat aortic ring (Fig. 5) assays, where EC1 domain in the Matrigel mimics cell–cell interactions and suppresses migration-dependent processes such as tubulogenesis, capillary growth, and sprouting. In summary, the key findings of this study are that T-cadherin down-regulates angiogenesis in vivo and in vitro and that the mechanism of its action lies in the inhibition of endothelial cell migration.

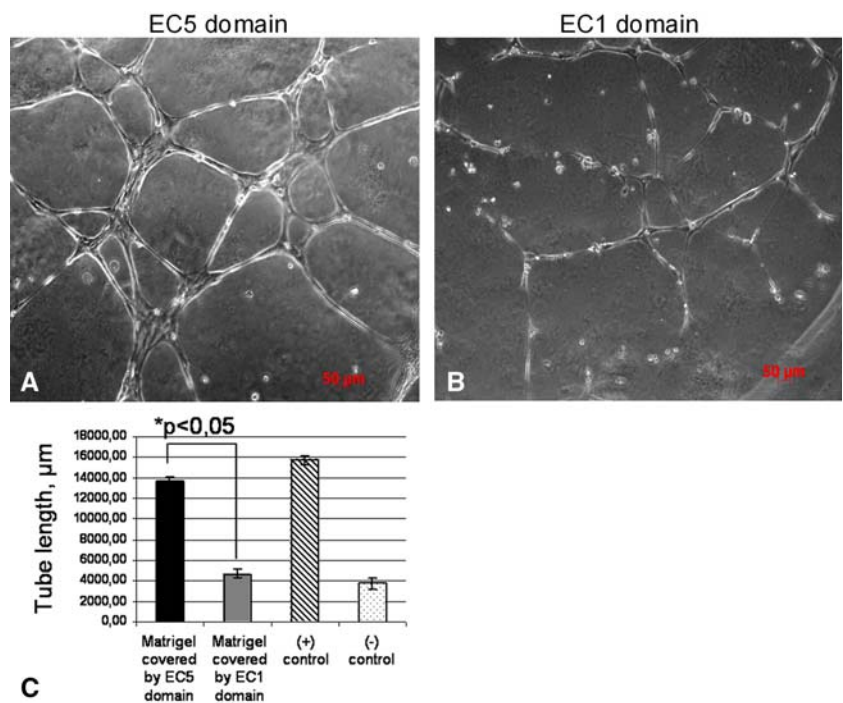
## Discussion

In normal adult organisms, most of the vasculature is quiescent. Excessive or insufficient vascular growth

**Fig. 3** Mature and nascent blood vessels in Matrigel plugs. (A, B) Double immunofluorescent staining of frozen Matrigel section with antibodies against endothelial marker CD31 (red fluorescence) and smooth muscle  $\alpha$ -actin (green fluorescence). (C, D) Double immunofluorescent staining of frozen Matrigel section with antibodies against endothelial marker CD31 (red fluorescence) and NG2 pericyte antigen (green fluorescence). (E) Vessel density of CD31-positive vascular structures,  $\alpha$ -actin-positive or both antigens-positive vessels on sections normalized to the DAPI-stained section area. (F) Vessel density of double positive CD31 and NG2 vascular structures on sections normalized to the DAPI-stained section area. Data present as mean  $\pm$  SEM of 42 sections. Data present as mean  $\pm$  SEM of 42 sections. \* $P < 0.05$ . Scale bars—20  $\mu$ m

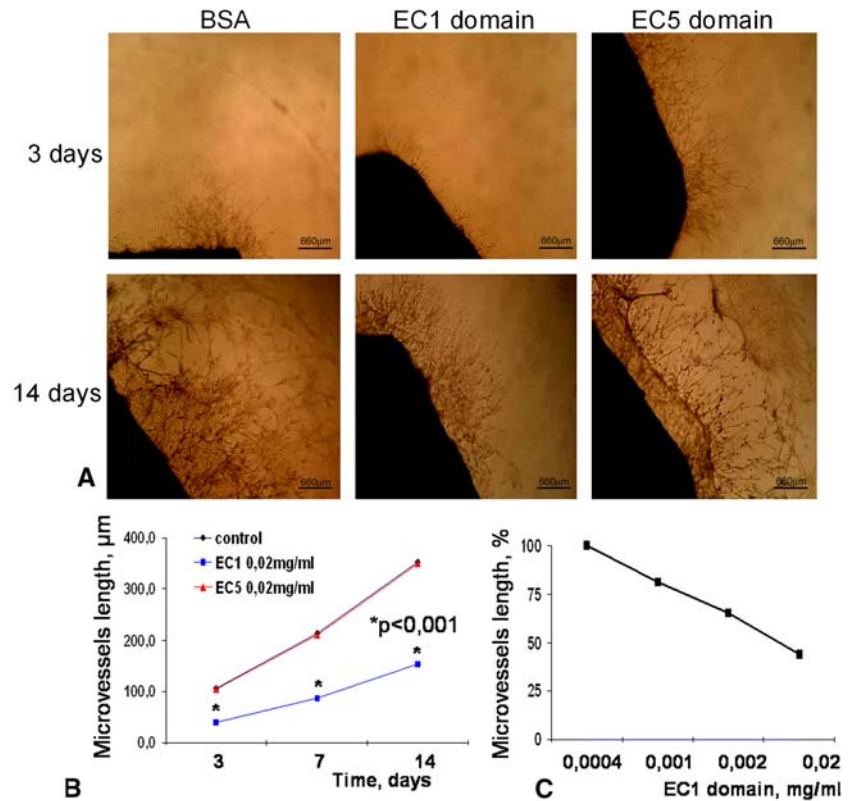


**Fig. 4** In vitro formation of capillary-like structures by HUVEC in Matrigel containing either EC1 or EC5 domains of T-cadherin. (A, B) Representative views of wells with HUVEC seeded on Matrigel with inclusion of indicated domains. (C) Average tube length calculated from four independent experiments performed in triplicates. Data presented as mean  $\pm$  SEM; \* $P < 0.05$ . Scale bars—50  $\mu$ m

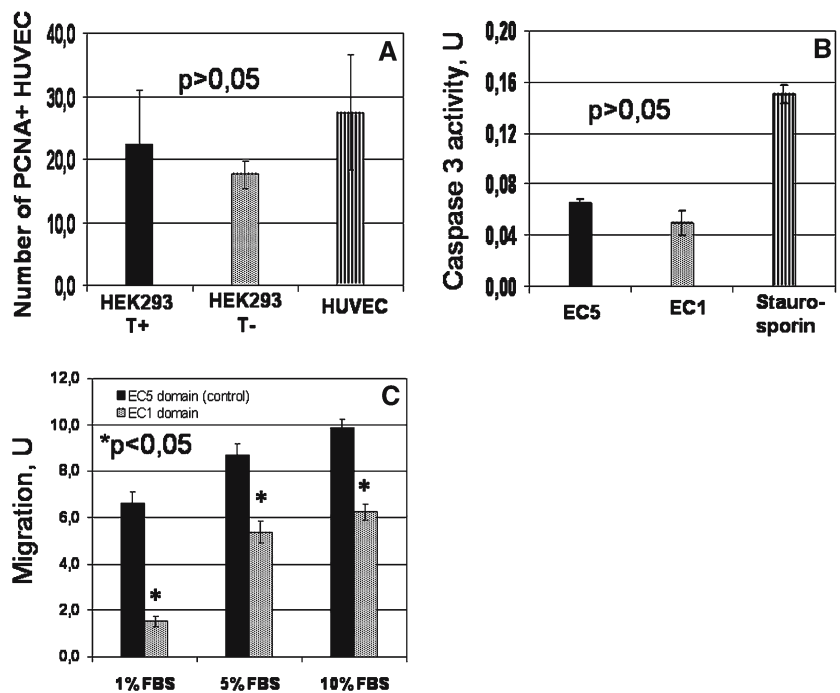




**Fig. 5** Suppression of vessel outgrowth by EC1 domain of T-cadherin from rat aorta rings. **(A)** Representative views of aorta rings placed in Matrigel with inclusion of indicated domains of T-cadherin after 3 and 14 days of incubation. **(B)** Average length of outgrowing microvessels calculated from 12 wells in four independent experiments. **(C)** Dose-dependent suppression of vessel outgrowth calculated in % from control in three independent experiments. Data presented as mean  $\pm$  SEM; \* $P < 0.001$ . Scale bars—650  $\mu$ m



**Fig. 6** Effects of T-cadherin on HUVEC. **(A)** Proliferation of HUVEC in co-culture with HEK cells overexpressing T-cadherin (HEK293+) or control cells (HEK293-) calculated by the number of PCNA-positive cells. **(B)** Caspase-3 activity in HUVEC cultured on EC1 precoated dishes as assessed by colorimetric assay. Data present as mean of three independent experiments performed in six repeats each  $\pm$ SEM;  $P = 0.05$ . **(C)** Migration of HUVEC in Boyden chamber through the membrane coated by recombinant domains of T-cadherin. EC1-aminoterminal extracellular domain of T-cadherin; EC5—juxtamembrane extracellular domain. Data presented as mean of three independent experiments performed in six repeats each  $\pm$  SEM; \* $P < 0.05$



contributes to non-neoplastic disorders, abnormal vessel remodeling, or tumor angiogenesis. Since tumor growth is dependent on recruiting new vessels, the inhibition of tumor angiogenesis is an effective method to suppress cancer [28, 29].

T-cadherin is endogenously expressed in the human vasculature in endothelial cells, smooth muscle cells, pericytes, and cardiomyocytes [9]. Moreover, T-cadherin expression is upregulated in a set of pathological conditions associated with excessive angiogenesis [9–12].

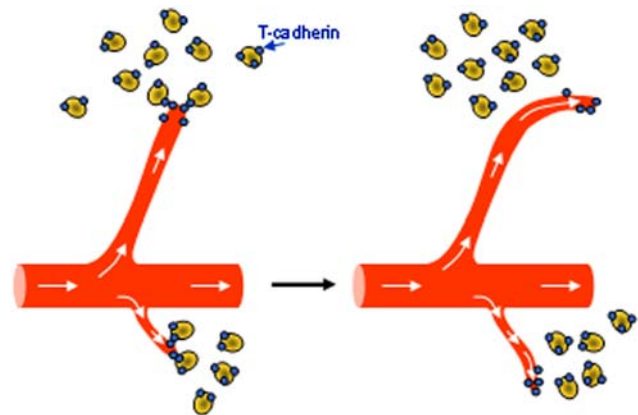
However, the physiological role of T-cadherin in vascular system remains largely elusive.

Our previous studies have implicated T-cadherin in vascular wall remodeling associated with excessive angiogenesis in restenosis and atherosclerosis [9, 10]. The present data provide the first evidence that T-cadherin specifically inhibits endothelial cell migration and capillary formation. This interaction requires endothelial cell contacts with T-cadherin expressing stromal cells or with immobilized EC1 domain, which mimics such cell–cell interactions [19, 30]. Our present data suggest that T-cadherin when abundantly expressed in microenvironment suppresses angiogenesis.

Overexpression of T-cadherin using adenoviral or plasmid transfection promotes HUVEC proliferation [19, 31, 32] and facilitates spontaneous cells migration of endothelial and other cells [14, 16]. In HUVEC, overexpression of T-cadherin protects cells from apoptosis and increases survival after exposure to oxidative stress [33]. These findings are in line with *in vivo* studies on T-cadherin expression in tumor vessels. In Lewis carcinoma lung metastasis, T-cadherin is upregulated in blood vessels penetrating the tumor, while in the surrounding tissue T-cadherin is not detected [11]. Similarly, in hepatocellular carcinoma (HCC) T-cadherin expression is increased in intratumoral capillary endothelial cells during neovascularization, whereas in the surrounding tumor tissue as well as in normal liver T-cadherin is not detected [12]. Together, this evidence indicates that T-cadherin expression in endothelial cells *in vitro* or in growing vessels *in vivo* correlates with enhanced angiogenesis. However, in these models the role of T-cadherin expression by stromal cells in neovascularization is not considered. In contrast, in our Matrigel model, L929 cells when injected into the plug, create the microenvironment with high level of T-cadherin. In these settings migrating endothelial cell, which naturally express T-cadherin contact T-cadherin positive stromal cells and this interaction results in suppressed blood vessel growth. This effect of T-cadherin is dependent upon the concentration of T-cadherin expressing cells injected into the Matrigel (data not shown). Consistently, suppression of vessel outgrowth in rat ring aorta assay is also dependent upon the concentration of EC1 domain in the Matrigel. These data suggest that homophilic interaction between T-cadherins on the cell surface could mediate the contact inhibition of endothelial cell invasion of the Matrigel stroma. This assumption has been further confirmed by quantitative and qualitative analysis of blood vessels of the Matrigel plugs. The amount of large blood vessels is not affected by the presence of T-cadherin in the Matrigel, while the number of small vessels and capillaries is dramatically reduced, suggesting that the early stages of neovascularization has been affected. Furthermore, small

vessels and capillaries having invaded the Matrigel plug with high level of T-cadherin do not express T-cadherin. Conceivably, T-cadherin expression in the stroma regulates qualitative composition of blood vessels infiltrating the tissue either by negative guiding of T-cadherin expressing vascular endothelial cells or by downregulation of T-cadherin expression in the growing vessels, or both. This would explain why T-cadherin was suggested to function as a tumor-suppressor factor [11, 34, 35] and why downregulation of T-cadherin in stroma cells of such tumors as basal cell carcinoma, cutaneous squamous carcinoma, non-small cell lung carcinoma (NSCLC), ovarian cancer, pancreatic cancer, and colorectal cancer [36–41] correlates with cancer progression.

The mechanism of T-cadherin mediated blood vessel growth suppression could be analogous to contact inhibition observed in the nervous system where T-cadherin acts as a negative guidance cue. There is now substantial evidence for a cross-talk between the vascular and nervous systems and that the same guiding molecules participate in neuronal and vascular growth [42]. At least four families of guidance molecules, which mediate repulsive or attractive behavior are known: netrins and their receptors DCC/neogenin and Unc5, slit ligands, and their roundabout (Robo) receptors, semaphorins, and their receptors plexin and neuropilin, ephrins and ephrin receptors [43, 44]. T-cadherin is another molecule, which guides blood vessels and growing axons through the complex environment to their targets by selecting pathways and choosing directions (Fig. 7). Ephrins and their Eph receptors are known to be involved both in guidance in the developing nervous system, and in embryonic and tumor angiogenesis [45, 46]. Interestingly, Ephrins A are also anchored to the plasma membrane via a GPI anchor [45, 46], suggesting a potential analogy between Ephrins A and T-cadherin. Interaction of ephrins with their receptors can result either in attraction, adhesion and invasion, or decreased adhesion and



**Fig. 7** Growing blood vessels avoid tissues expressing T-cadherin

repulsion. The mechanisms of GPI-anchored ephrin signaling are still obscure but probably involve ligand clustering within lipid rafts and interaction with adaptor proteins. Even less is known about T-cadherin signaling partners, yet some signaling mechanism of GPI-anchored Ephrins A and T-cadherin could be similar.

In the developing nervous system T-cadherin expresses neural crest cells, growing axons, and axons projecting through the somitic sclerotome avoid T-cadherin expressing tissues [6, 47, 48]. Thus, we speculate that T-cadherin acts as a negative guidance cue and that the general mechanism of T-cadherin mediated repulsion could involve homophilic T-cadherin interaction and contact inhibition. This assumption has been confirmed in our *in vitro* experiments utilizing immobilized EC1 domain, which is responsible for homophilic T-cadherin interaction of contacting cells [19]. The migration of endothelial cell, which endogenously express T-cadherin [16] is inhibited by immobilized EC1 in Boyden chamber assay, ring aorta, and capillary tube *in vitro* assays in contrast to soluble T-cadherin domains. *In vivo* T-cadherin expression in the microenvironment of Matrigel plugs inhibits the ingrowth of small vessels and capillaries but does not affect pericyte recruitment and the maturation of blood vessels. Dynamic evaluation of hemoglobin content in Matrigel supports the assumption that T-cadherin inhibits angiogenesis by suppressing directed endothelial cell migration and vessel invasion into the plug. Interestingly, T-cadherin does not affect endothelial cell proliferation, adhesion, or apoptosis.

Our data demonstrate for the first time that T-cadherin expression in the microenvironment suppresses the growth of blood vessels. Tumor stroma is not just an inert environment [49] and “activated fibroblasts” of the tumor at the site of metastasis promote cancer cell proliferation and also support the initiation of angiogenesis; while normal fibroblasts, when injected into the tumor stroma can inhibit cancer progression [50]. Thus, application of cells constitutively expressing T-cadherin might serve as a novel therapeutic approach in cancer.

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## Appendix

It was previously shown that T-cadherin could mediate weak homophilic adhesion in aggregation assays *in vitro* [51, 52]. To analyze whether T-cadherin is involved in adhesion that underlies cell sorting, we performed

time-lapse live-cell fluorescence imaging. For these experiments we co-cultured control HEK293 cells with HEK293 cells co-expressing GFP and T-cadherin [14]; or CellTracker labeled control L929 cells with L929 cells expressing T-cadherin. Cells intermingled equally and we could detect no difference in the number, size, or distribution of fluorescently labeled cells in the aggregates (data not shown). There was also neither cell sorting nor T-cadherin-mediated repulsive or adhesive effects observed over a period of 2–4 days in time-lapse living cell experiments (Figure I, movie).

## Materials and methods

### Time-lapse live-cell fluorescence imaging

Time-lapse observations were performed using a DeltaVision Olympus IX70 inverted microscope with a  $\times 20/0.40$  NA objective lens with relief contrast optics. Cells were suspended with cell-dissociation buffer (Invitrogen, Gibco) before the experiment and plated in fibronectin-coated Lab-Tek Chambered #1.0 coverglass systems in 1:1 proportion. The total concentration of cells was  $35,000/\text{cm}^2$ . The cells were observed during 2–4 days in a  $37^\circ\text{C}$  environmental chamber. Each image was acquired with 4-min exposure of the CCD camera.

### Labeling of cells with CellTracker reagent

Cells were labeled using CellTracker Green CMFDA reagent (Invitrogen, Molecular Probes) according to manufacturer instructions. In brief, the cells were incubated with  $10\ \mu\text{M}$  CMFDA for 15 min followed by incubation in fresh pre-warmed medium for another 30 min at  $37^\circ\text{C}$ .

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