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The bHLH TAL-1/SCL regulates endothelial cell migration and morphogenesis

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Summary

The basic helix-loop-helix *tal-1* gene (or *scl*), known for its fundamental role in embryonic and adult hematopoiesis in vertebrates, is also required for embryonic vascular remodeling. In adults, TAL-1 protein is undetectable in quiescent endothelium but it is present in newly formed vessels including tumoral vasculature, indicating its involvement in angiogenesis. Here, we demonstrate that TAL-1 expression is tightly regulated during *in vitro* angiogenesis: it is low during the initial step of migration and is upregulated during formation of capillary-like structures. We investigated whether ectopic expression of either wild-type TAL-1 or a dominant-negative mutant lacking the DNA-binding domain (Δ -bas) modulates the activity of human primary endothelial cells in the angiogenic processes of migration, proliferation and cell morphogenesis. Overexpression of either wild-type or Δ -bas TAL-1 affected chemotactic migration of primary

endothelial cells without modifying their proliferative properties. Ectopic expression of wild-type TAL-1 accelerated the formation of capillary-like structures *in vitro* and, *in vivo*, enhanced vascularisation in mice (Matrigel implants) associated with a general enlargement of capillary lumens. Importantly, transduction of the mutant Δ bas completely impaired *in vitro* angiogenesis and strongly inhibited vascularisation in mice. Taken together, our data show that TAL-1 modulates the angiogenic response of endothelial cells by stimulating cell morphogenesis and by influencing their behavior in migration. This study highlights the importance of TAL-1 regulation in postnatal vascular remodeling and provides the first physiological evidence that links TAL-1 activity to endothelial cell morphogenic processes.

Key words: TAL-1/SCL, bHLH, Angiogenesis, Endothelial cells

Introduction

The vascular system is one of the first organ systems to develop in close association with the hematopoietic lineage in vertebrate embryos. Vasculogenesis and angiogenesis are the two major processes responsible for the formation of blood vessels. Vasculogenesis refers to *in situ* formation of primitive blood vessels from endothelial precursors or angioblasts (for a review, see Risau and Flamme, 1995). Angioblasts migrate extensively to distant sites in the embryo, where they proliferate and differentiate to form a primitive tubular network, which includes some of the major vessels, such as the aorta and major veins. The primary vascular plexus is then remodeled to form new capillaries by the sprouting and/or growing of existing vessels, a process termed angiogenesis (Risau, 1997). Endothelial precursor cells (EPCs) have been identified in adult bone marrow and peripheral blood as well as in umbilical cord blood (Kalka et al., 2000; Murohara et al., 2000). These EPCs have been shown to share properties with embryonic angioblasts (Asahara et al., 1997; Asahara et al., 1999); they have the capacity to migrate, proliferate and differentiate *in situ* into mature endothelial cells and to colonize sites of active neovascularization.

Angiogenesis is required for a number of physiological situations in adult life such as pregnancy, menstrual cycle, and tissue repair. Neoangiogenesis is also observed under

pathological conditions such as tumors, inflammatory disorders, atherosclerosis and diabetes. In adults, endothelial cells normally remain quiescent, show a low turnover rate and rarely proliferate. However, following an appropriate stimulus, endothelial cells are activated and participate in angiogenesis. Members of the vascular endothelial growth factor (VEGF) and angiopoietin families are considered to be key angiogenic mediators contributing to both physiological and pathological angiogenesis (for a review, see Gale and Yancopoulos, 1999).

Despite recent progress in the identification of signaling molecules and cell surface receptors that regulate blood vessel formation, little is currently known about the precise nuclear transcriptional events that govern endothelial fate. Gene knockout in mice has allowed the identification of several transcription factors such as ETS-1 (Lelievre et al., 2001), TEL (Wang et al., 1997), FLI (Hart et al., 2000) and the Lim-only protein LMO-2 (Yamada et al., 2000), as potential mediators of angiogenesis.

Several basic helix-loop-helix factors, or bHLHs, (Henderson et al., 2001; Visvader et al., 1998; Yamagishi et al., 2000) and their inhibitors, the Id protein family (Lyden et al., 1999; Lyden et al., 2001) have been shown to participate in the formation and/or maturation of the vascular system. Initially identified by us and others as an oncogene in human T cell leukemia (Aplan et al., 1990; Baer, 1993; Begley et al., 1989;

Bernard et al., 1990), the bHLH gene *tal-1*, also named *scl*, was found to be essential for normal embryonic and definitive hematopoiesis (Porcher et al., 1996; Robb et al., 1995; Shivdasani et al., 1995) as well as for proper vascular development (Visvader et al., 1998); *tal-1*^{-/-} embryos, rescued for *tal-1* expression in primitive hematopoietic cells, exhibit defective yolk sac angiogenesis, presumably due to an intrinsic defect in *tal-1*^{-/-} endothelial cells (Visvader et al., 1998). It also appears that *tal-1* activity may be important for the commitment of the common precursor of both hematopoietic and endothelial lineages, the hemangioblast. During embryogenesis, *tal-1* is one of the first markers detected in isolated mesodermal cells that give rise to angioblasts while its expression is down-regulated in the endothelium of mature vessels (Drake et al., 1997; Drake and Fleming, 2000). Overexpression of *tal-1* in zebrafish embryos leads to an increase in both hematopoietic and endothelial markers (Gering et al., 1998). Furthermore, *tal-1*^{-/-} ES cells are unable to generate hemangioblast-derived blast colonies, but do give rise to colonies that appear to represent a novel stage in the transition from mesoderm to hematopoietic and endothelial lineage commitment (Robertson et al., 2000). In adults, *tal-1* expression in non-hematopoietic cells is restricted to endothelial cells lining new blood vessels (Kallianpur et al., 1994; Pulford et al., 1995). Furthermore, TAL-1 has been proposed to modulate, in combination with GATA and ETS transcription factors, the *Flk-1* gene that is known to play a pivotal role in both vasculogenesis and angiogenesis (Kappel et al., 2000). Finally, TAL-1 protein is expressed at high levels in the vasculature of human tumors but not in mature quiescent vasculature (Chetty et al., 1997). Based on these observations, *tal-1* has been proposed to regulate molecular and cellular events involved in both vasculogenesis and angiogenesis.

In the present study, we have investigated the role of TAL-1 in the different processes of adult angiogenesis. TAL-1 expression was modulated in primary human endothelial cells using adenoviral vectors transducing wild-type or dominant-negative mutant TAL-1 protein, or knocked down by RNA interference, and the effects on migration, proliferation, and in vitro and in vivo angiogenesis were assessed.

Materials and Methods

Endothelial cell cultures

Primary human endothelial cells (ECs) from umbilical vein (HUVECs) were obtained from Clonetics (BioWhittaker, USA) and were propagated through passage 6 in MCDB 131 medium (Gibco-BRL) supplemented with 2 mM Glutamax (Gibco-BRL), 10% fetal calf serum (FCS), 10 U/ml porcine heparin (Sigma), 10 ng/ml hu-EGF (PeproTech, Inc. USA), 35 µg/ml endothelial cell growth supplement (ECGS, BD, Biosciences) and 1 µg/ml hydrocortisone (Sigma). The immortalized cell lines, HMEC-1 (Ades et al., 1992) and BMEC-1 (Candal et al., 1996) were obtained from the Center for Disease Control (CDC, Atlanta, USA) and cultured in the same medium as HUVEC without ECGS. ECV304 cells were obtained from the American Tissue Culture Center (ATCC, Bethesda, MD) and cultured in M199 with 10% FCS.

CD34 + cell separation and expansion of UCB-endothelial cells

Mononucleated cells (MNCs) were isolated from umbilical cord blood using the Histopaque-density centrifugation method. CD34-positive

cells were separated from 1-2×10⁸ MNCs by positive selection using CD34 monoclonal antibody coupled to magnetic beads (MACS, Miltenyi Biotech). 1-2×10⁶ CD34+ cells were plated on gelatin-coated tissue culture flasks in endothelial-specific medium supplemented with 10% FBS and appropriate cytokines (ECGF, bFGF and EGF). Non-adherent cells were eliminated by regularly changing the medium during the first 6 days of culture. By 3-4 weeks, the cells formed a monolayer with a typical endothelial spindle-shaped morphology; FACS analysis indicated that the majority of the cells expressed the specific endothelial markers, VE-cadherin and CD31 (85% and 95%, respectively).

Adenovirus construction and production

Recombinant adenoviruses were generated using the Adeno-X™ Tet-Off™ expression system (Clontech, USA) following the instructions of the manufacturer. Ad-TRE-wtTAL-1 contains a 1.35 kilobase fragment which is the entire human *TAL-1* wild-type coding sequence (Leroy et al., 1995). Ad-TRE-Δbas contains a 1.1 kilobase *EcoRI* fragment derived from pCSCLΔB (Aplan et al., 1990) in which the sequences encoding the basic region of human TAL-1 protein have been deleted (amino acids 188-199).

Recombinant defective adenoviruses were produced by transfection of human embryonic kidney (HEK) 293 cells. After serial amplifications in 293 cells, adenoviral particles were purified by two successive ultracentrifugations through a CsCl gradient. Virus stocks were resuspended in 3% sucrose in phosphate-buffered saline (PBS), aliquoted and stored at -80°C prior to infection. Independent productions were carried out in the laboratory and by 'The Vector Core of the Hospital of Nantes' supported by the Association Française contre les Myopathies (AFM).

Transduction of endothelial cells

Exponentially growing ECs were seeded onto collagen I-coated dishes. After 4 hours at 37°C, ECs were co-infected with Ad-Tet-Off and either Ad-TRE-wtTAL-1, Ad-TRE-Δbas or control Ad-TRE-LacZ (ratio 2:1) at a multiplicity of infection of 100 for HUVECs and of 200 for UCB-ECs in endothelial-specific complete medium. Biological tests (in vitro angiogenesis, cell migration and proliferation) and preparation of whole cell lysates were carried out between 20 and 24 hours after adenoviral infection.

Matrigel-based in vitro angiogenesis

Wells of a 24-well plate were coated with Matrigel (BD Biosciences) and incubated at 37°C for 30 minutes. 1×10⁵ cells (HUVECs), 8×10⁴ cells (UCB-ECs) or 2×10⁵ cells (HMEC-1) in complete EC medium were added to Matrigel-coated wells and incubated at 37°C with 5% CO₂ for 24 hours. When required, cells were recovered from Matrigel and treated with Matrisperse (BD Biosciences) in the presence of a 5-fold concentrated cocktail of protease inhibitors (Boehringer Mannheim). After treatment, cells were centrifuged and washed with PBS for preparation of cell extracts.

Western blot analysis

Western blots were performed as described previously (Leroy et al., 1995). Membranes were probed with the monoclonal anti-TAL-1 antibody 3 BTL 73 (Pulford et al., 1995) using a goat anti-mouse IgG/horseradish peroxidase conjugate (Amersham) as the secondary antibody. Bands were developed using a chemiluminescent substrate (ECL Plus, Amersham). As a control for loading and normalization, membranes were re-probed with an anti-GAPDH rabbit antiserum (kindly provided by Jean-Marie Blanchard, France). Bands were scanned with Snaps Scan 1236 (AGFA) and quantified using the program Scion.

Total RNA preparation and real-time PCR analysis

Total RNA was extracted using the High Pure RNA Isolation kit (Roche Diagnostics) following the manufacturer's instructions. Reverse transcription using 1.0 µg total RNA was performed with SuperScript II reverse transcriptase (Gibco-BRL) and oligo(dT) priming. The primer sequences of the oligonucleotides used for PCR were as follows:

tal-1 forward: 5'-CTCGGCAGCGGGTCTTTGG-3'
tal-1 reverse: 5'-GCCTGCTTACCACCTTCTTG-3'
GAPDH forward: 5'-ACAGTCCATGCCATCACTGCC-3'
GAPDH reverse: 5'-GCCTGCTTACCACCTTCTTG-3'

PCR amplification reactions were performed in a DNA thermal cycler (Light cycler ROCHE). DNA was denatured at 95°C, followed by 45 PCR cycles; each cycle included denaturation at 95°C, primer annealing for 10 seconds at 65°C (for *tal-1*) or 5 seconds at 70°C (for *GAPDH*) and a final elongation step of 15 seconds at 72°C. PCR products were analyzed using the Light Cycler ROCHE program.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously (Courtes et al., 2000). Each 10 µl reaction mixture contained, 3 or 10 µg of nuclear extract, 10 mM Hepes (pH 7.8), 50 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5 µg of poly(dIdC) and 100,000 cpm of a ³²P-labeled double-stranded oligonucleotide probe containing the TAL-1 E-box sequence (5'-ACCTGAACAGATG-GTCGGCT-3'). For supershift, the following antibodies were used: mAb anti-TAL-1 (3BTL 73), mAb anti-E47 (BD Pharmingen) or the irrelevant 12CA5 mAb (anti-HA).

In vivo assessment of angiogenesis

500 µl ice-cold Matrigel (9-10 mg/ml; BD Biosciences) mixed with heparin (10 U/ml; Sigma), and bFGF (250 ng/ml; Peprotech, Inc. USA) were injected subcutaneously into 8- to 16-week-old male SCID mice at sites near the abdominal midline, with three injections per mouse. Injection sites were chosen such that each animal received the three different recombinant Ad-TRE: -LacZ, -wtTAL-1, -Δbas along with the Ad-Tet-Off (3×10⁸ total infectious particles; ratio Ad-Tet-Off/Ad-TRE: 2:1). The mixture of adenoviral vectors was either incorporated in the Matrigel prior to subcutaneous implantation in the abdomen of SCID mice or injected within the plug 1 day after implantation. Animals were sacrificed 5 or 7 days after injection; implants together with associated skin were recovered and immediately photographed.

For histological analysis, implants were fixed in 3.7% buffered formaldehyde in PBS, embedded in paraffin wax, sectioned, deparaffined and stained with Hematoxylin and Eosin. For immunohistochemical analysis, Matrigel plugs were embedded in Cryomatrix compound (Thermo Shandon, USA) and snap frozen in liquid nitrogen. Frozen implants were then sectioned in 5 µm sections, fixed with 3.7% buffered formaldehyde in PBS and stained with anti-CD31 rat mAb (clone MEC13.3, Pharmingen). Bound rabbit anti-rat secondary biotinylated Ab was visualized using Extravidin horseradish peroxidase (Sigma Chemical Co).

Migration assays

Migration assays were performed as described previously (Levy et al., 2002). The cells that had migrated through the inserts were fixed in 3.7% formalin, stained with propidium iodide and analyzed under a fluorescence microscope. The cells that had spread on the lower side of the inserts were counted on photographs of six random fields per well (original magnification, ×10).

SiRNA preparation and transfection

SiRNAs targeting two sites in the *tal-1* mRNA, chosen on the basis

of the criteria outlined in Elbashir et al. (Elbashir et al., 2001), were prepared by in vitro transcription using the Silencer siRNA construction kit (Ambion, Austin Texas, USA). Preliminary results (not shown) demonstrated that gene silencing was more efficient with one of the two targets (nt 879-897) (Bernard et al., 1991). This sequence was targeted in further experiments with a chemically synthesized siRNA (Eurogentec, Belgium).

tal-1 sense strand: 5'-GAAGCUCAGCAAGAAUGAGTT-3'
tal-1 antisense strand: 5'-CUCAUUCUUGCUGAGCUUCTT-3'

A chemically synthesized siRNA targeting an irrelevant sequence was used as a control:

Control sense strand: 5'-GUGUUGGGUCGCGAAAGGCTT-3'
 Control antisense strand: 5'-GCCUUUCGCGACCCAACACTT-3'
 (The 3' TT in all strands was DNA.)

The day before transfection, HUVECs were plated onto 60 mm dishes coated with gelatin, at 30-35% confluency in complete EC medium. Two successive transfections of siRNA (1.0 µg per 60 mm dish) at 24-hour interval were carried out with Lipofectamine Plus (Invitrogen). Cells were analyzed 24-48 hours after the second transfection.

Statistical analysis

All results were expressed as the mean±s.d. Significance of differences was determined with the Student's *t*-test with significance at *P*<0.05.

Results

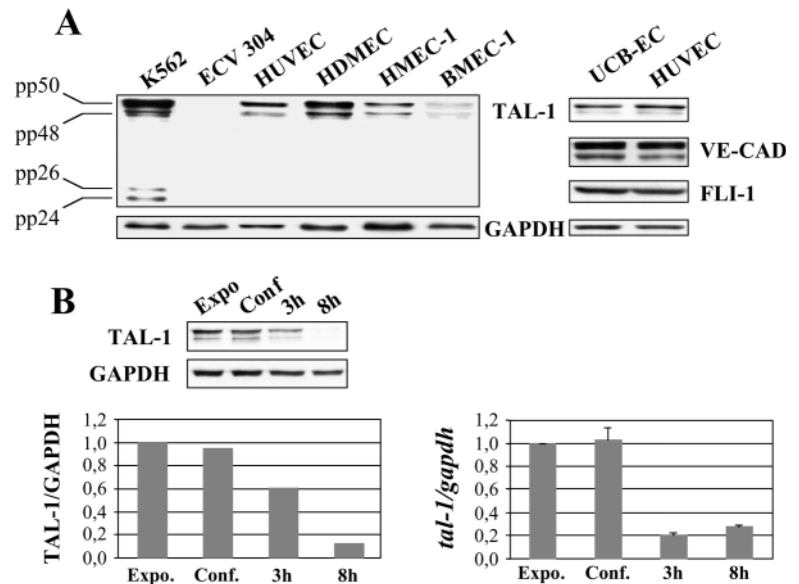
TAL-1 protein expression in exponentially growing endothelial cells

tal-1 encodes two major phosphorylated proteins (pp48/50 and pp24/26) that share the same C terminus containing the bHLH motif (Bernard et al., 1991) but differ in the presence of a transactivation domain in the N terminus of pp48/50 (Sanchez and Rabbitts, 1994). TAL-1 protein expression was examined in a variety of human ECs by immunoblotting with the 3BTL-73 mAb which recognizes all TAL-1 isoforms (Pulford et al., 1995). Expression levels of the ubiquitous GAPDH protein were examined as a control for loading differences. TAL-1 proteins were detected in all ECs tested but, in contrast to hematopoietic cells (K562), pp48/pp50 were the only TAL-1 isoforms detected in ECs (Fig. 1A, left). As expected, no TAL-1 protein was detected in the ECV 304 epithelial bladder carcinoma cell line. TAL-1 expression levels were quite variable, ranging from nearly undetectable in ECs derived from bone marrow (BMEC-1) to high in ECs derived from microvessels (HDMEC). Circulating endothelial precursor cells isolated from cord blood were analyzed after 25 days in culture (UCB-EC, Fig. 1A, right); at this time, a large majority of the cells expressed endothelial-specific markers (see Material and Methods). Similar levels of VE-cadherin and FLI-1 expression were found in UCB-EC cell extracts as in HUVECs, confirming the endothelial nature of UCB-ECs. pp48/pp50 TAL-1 were also present in UCB-ECs.

tal-1 expression is reduced in non-proliferating ECs in the absence of endothelial-specific growth factors

Several studies have reported that quiescent human and murine endothelia do not express *tal-1* (Chetty et al., 1997; Kallianpur et al., 1994). Therefore, we analyzed *tal-1* expression in non-proliferating ECs. Exponentially growing HUVECs were

Fig. 1. TAL-1 protein expression in human endothelial cells. (A) pp48-50 is the only TAL-1 protein species detected in human proliferating endothelial cells (ECs). (Left) TAL-1 expression was assessed in whole cell lysates (30 µg) by immunoblotting with the 3BTL73 monoclonal antibody directed against the C terminus of the human TAL-1 protein (top). The blot was reprobed with an anti-GAPDH rabbit antiserum to evaluate protein loading. Expression was assessed in the following cells: K562, a human erythroleukemic cell line; ECV 304, an epithelial cell line established from a human bladder carcinoma; HUVEC, ECs derived from umbilical cord; HDMEC, ECs derived from dermal microvessels; HMEC-1, a cell line derived from HDMECs immortalized with large T antigen (Ades et al., 1992) and BMEC-1, a cell line established from bone marrow ECs immortalized with large T antigen (Candal et al., 1996). (Right) Expression of TAL-1 protein was assessed in circulating endothelial cells derived from umbilical cord blood (UCB-EC) at day 25 of culture (see Materials and Methods). The blot was reprobed for FLI-1 and VE-cadherin protein expression, two endothelial-specific markers, and for GAPDH to monitor loading. (B) Down-regulation of *tal-1* expression in quiescent non-activated HUVECs. Proliferating HUVECs (Expo) were grown to confluence and were maintained either in complete medium for an additional 8 hours (Conf) or in medium containing only 5% FCS for 3 hours and 8 hours. (Left) Whole cell lysates were prepared at the indicated times after starvation and TAL-1 expression was monitored by western blot analysis using the 3BTL73 anti-TAL-1 monoclonal antibody. The same blot was reprobed with an anti-GAPDH rabbit antiserum for normalization of protein levels. TAL-1 and GAPDH proteins were both quantified by densitometry and TAL-1 protein content relative to GAPDH in one of two representative experiments is shown. The TAL-1 content from exponentially growing HUVECs (Expo) was arbitrarily set at 1. (Right) *tal-1* mRNA expression was monitored by real-time PCR. The *tal-1* mRNA signal was normalized to that of *GAPDH*. Data shown are the means \pm s.d. of three independent RT-PCR experiments. *tal-1* mRNA content from exponentially growing HUVECs was arbitrarily set at 1.



brought to confluence in complete endothelial-specific medium. Thereafter, the medium was replaced by M199 medium containing 5% serum without EC-specific growth factors. Total cell lysates and total RNA were prepared at different time points and *tal-1* expression was analyzed. The kinetics of TAL-1 protein expression in HUVEC maintained in the absence of endothelial-specific growth factors demonstrated a 60% and 80% decrease after 3 and 8 hours of starvation respectively (Fig. 1B, left). The decrease in TAL-1 expression was not specific to the confluent nature of the cells as maintenance of these cells in complete endothelial-specific medium for an additional 24 hours resulted in a slight increase in TAL-1 protein content (about 1.5- to 2-fold as compared to exponentially growing ECs; data not shown).

To investigate whether TAL-1 down-regulation was associated with decreased mRNA levels, real-time PCR analysis was performed using the Light Cycler System (Roche) and *GAPDH* expression was used for normalization. As shown in Fig. 1B (right), *tal-1* mRNA levels were decreased by 80% following a 3-hour culture without endothelial-specific growth factors as compared to exponentially growing cells. Thus, this phenomenon is associated with diminished *tal-1* mRNA and protein levels.

Modulation of TAL-1 protein during in vitro angiogenesis

In vitro angiogenesis operates on the principle that ECs form capillary-like structures when cultured on a supportive matrix derived from a murine tumor (Matrigel). This assay reproduces the angiogenic processes of migration, alignment and cell differentiation, and has proved to be an important tool for

studying the mechanisms of angiogenesis (Cid et al., 1993; Nicosia and Ottinetti, 1990).

A typical in vitro angiogenesis experiment is shown in Fig. 2A. ECs plated on Matrigel underwent alignment and elongation within 1-3 hours after seeding, establishing the pattern for further capillary networking. By 6 hours, formation of cords had begun and by 12 hours virtually all cells had fused into continuous cords. Stabilization and refinement of the cords progressed until 24 hours. We studied the kinetics of endogenous TAL-1 expression during in vitro angiogenesis of human ECs. Fig. 2B illustrates an immunoblot analysis from one typical experiment with HUVECs. Whereas a small decrease in TAL-1 was detected within the first hours (5 hours), a significant increase in TAL-1 protein levels was repeatedly observed during the step of the cord-maturation process (12-24 hours), 3- to 5-fold higher than that detected in exponentially growing cells (Fig. 2B). Similar experiments were carried out with UBC-ECs (Fig. 2C) and immortalized HDMECs (HMEC-1 cells; fig. 2D) and in a similar manner to HUVECs, TAL-1 protein decreased by 3 or 5 hours (60% and 80% reduction respectively), followed by a progressive increase, which reached a maximum at 24 hours when the capillary-like network was formed.

Altogether, our data show that TAL-1 expression is modulated during in vitro angiogenesis: down regulated within the early steps of migration and upregulated during tubule-like morphogenesis process.

Effects of overexpression of wild-type or mutant TAL-1 protein on in vitro angiogenesis

The data described above suggest that a transient increase in

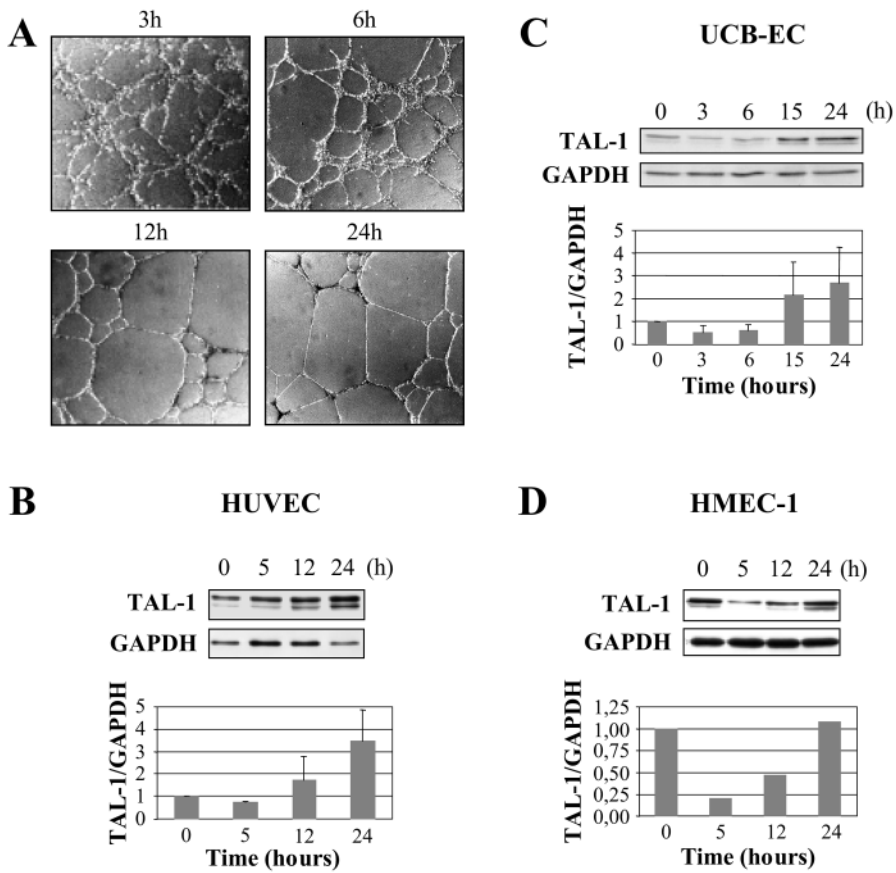


Fig. 2. *tal-1* expression is modulated during in vitro angiogenesis. Exponentially growing cells (HUVECs, HMEC-1 or UCB-ECs) were plated on Matrigel and cultured for 24 hours.

(A) Photomicrographs of two-dimensional cultures of HUVECs on Matrigel demonstrating the progression of the tubular network. Initial plating on Matrigel corresponds to time 0 hour. (B-D) Cells were recovered from Matrigel at the indicated times to prepare whole cell extracts and TAL-1 expression was monitored by western blot analysis using the 3BTL73 anti-TAL-1 monoclonal antibody. The blots were reprobbed with an anti-GAPDH rabbit antiserum for normalization of protein levels. TAL-1 and GAPDH proteins were both quantified by densitometry. TAL-1 content from exponentially growing ECs was arbitrarily set at 1. Representative autoradiographs of three independent experiments and the means \pm s.d. of TAL-1 protein content relative to that of GAPDH are shown. (B) HUVECs; (C) UCB-ECs at day 30-33 of culture; (D) HMEC-1.

TAL-1 levels might be functionally important in the regulation of tube-like formation. To better assess the potential role of TAL-1 in this process, we overexpressed in primary human ECs wild-type TAL-1 protein (wt) or a dominant negative mutant TAL-1 (Δ bas), which is not able to interact with DNA.

Recombinant adenoviral vectors were used to mediate gene transfer; the transgenes encoding TAL-1 proteins were placed under the control of tetracycline responsive elements (Ad-TRE-) and a minimal cytomegalovirus promoter (Adeno-X Tet-Off; Clontech). In this system, transgene expression is activated by co-infection of an adenovirus constitutively expressing the tetracycline repressor (Ad-Tet-Off). Using these recombinant adenoviruses, we routinely observed high efficiency of infection of human ECs (essentially 100% at a m.o.i. of 100 for HUVECs and of 200 for UCB-ECs) as assessed by co-infection with the control adenovirus transducing the β -galactosidase gene (Ad-TRE-LacZ).

The ectopic expression of both wt and Δ bas TAL-1 proteins was systematically confirmed by western blot analysis. As illustrated in Fig. 3A, HUVECs transduced with either TRE-wtTAL-1 or TRE- Δ bas overexpressed the ectopic protein about 100-fold over endogenous TAL-1 levels. In hematopoietic cells, TAL-1 proteins have been shown to bind a specific E-box DNA motif (AACAGATGGT) as heterodimers with ubiquitous bHLH (E proteins), preferentially with the E47 isoform encoded by the E2A gene (Hsu et al., 1994a; Hsu et al., 1994b). Therefore, we assessed the DNA-binding activity of endogenous TAL-1 in ECs by electrophoretic mobility shift assays (EMSA). Nuclear extracts (10 μ g) from non-infected

HUVECs were incubated with a DNA probe containing the specific E-box sequences. In addition to the major complexes, which contain homo- and heterodimers of E proteins, EMSA revealed a slower migrating specific complex which contained TAL-1/E47 heterodimers as demonstrated by an anti-TAL-1 mAb-induced supershift and reduced complex in the presence of the anti-E47 antibody (Fig. 3B, left). Thus, in endothelial cells, TAL-1 interacts with E47 to bind the TAL-1 E-box motif.

The DNA-binding properties of the exogenous TAL-1 were controlled by EMSA experiments. Ad-wtTAL-1-infected HUVECs overexpressed active TAL-1 as demonstrated by a significant increase in TAL-1/E-DNA complex formation in nuclear extracts (Fig. 3B, right). As expected, no TAL-1/E DNA complex was detected in Ad- Δ bas-infected HUVECs. Complexes formed by endogenous TAL-1 were not detected in control Ad-LacZ-infected HUVECs, because of the experimental conditions (only 3 μ g of nuclear extracts were used in these assays).

Twenty hours post-infection, transduced ECs were plated onto Matrigel and incubated for an additional 24 hours; a typical experiment with HUVECs is shown in Fig. 3C. At 90 minutes after seeding, most of the Ad-LacZ- and Ad-wtTAL-1-infected ECs had lost their cobblestone morphology and had formed chains of cells. Repeatedly, the cytoplasmic extensions that precede cord formation occurred earlier and were more numerous in wtTAL-1-transduced ECs as compared to LacZ-control ECs (Fig. 3C, 90 minutes). In contrast, Δ bas-transduced cells displayed a very poor alignment and developed very few cytoplasmic extensions. At 20 hours, transduction of wtTAL-1 did not result in gross modifications in tubule-like network as compared to control infected cells. In striking contrast, Ad- Δ bas transduction provoked a complete disorganization of capillary-like structures. Similar observations were made with UCB-ECs (not shown).

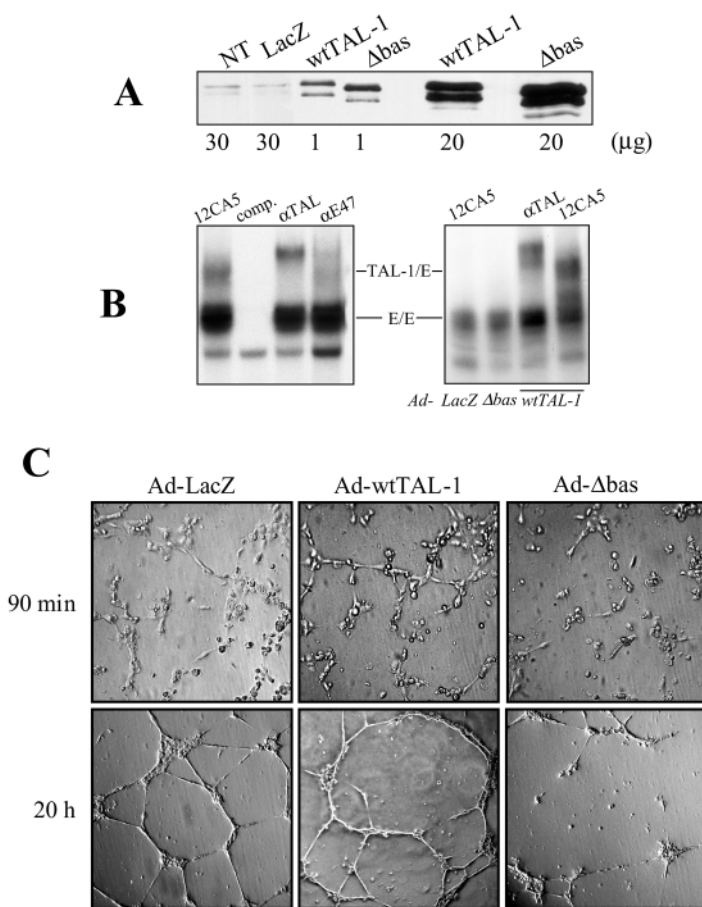


Fig. 3. Effect of wild-type and Δ bas TAL-1 overexpression on in vitro angiogenesis. (A) Western blot analysis showing ectopic overexpression of wild-type (wt) and Δ bas TAL-1 protein in adenovirus-infected HUVECs. ECs were co-infected with Ad-Tet-Off and one of the following: Ad-TRE-wtTAL-1, Ad-TRE- Δ bas or the control Ad-TRE-LacZ. Whole cell lysates were prepared 20 hours after infection. Note the intensities of wt and Δ bas TAL-1 detected in 1 μ g of whole cell lysate derived from infected cells as compared to that of endogenous TAL-1 detected in 30 μ g of whole cell lysate derived from Ad-LacZ-infected cells (LacZ) or non-treated cells (NT). (B) Electrophoretic mobility shift analysis (EMSA) of TAL-1 DNA binding activity. A double stranded 32 P-labeled oligonucleotide probe containing the TAL-1 E-box consensus sequence was incubated with nuclear extracts from HUVECs. (Left) EMSA showing the binding of TAL-1/E endogenous heterodimers to the TAL-1 E-box in nuclear extract from HUVEC (10 μ g). The specificity of the interaction was controlled by adding a 100-fold excess of unlabeled TAL-1 E-box oligonucleotides (comp). Additionally, the presence of TAL-1/E heterodimers was verified by supershifting the complexes with an anti-TAL-1 antibody (α TAL) or disrupting them with an anti-E47 antibody (α E47). An irrelevant monoclonal antibody (12CA5) was added as a negative control. (Right) Ectopic TAL-1 DNA binding on E-box sequences was monitored in the presence or absence of a supershifting anti-TAL-1 mAb. Experimental conditions precluded the detection of endogenous TAL-1/E heterodimers in control LacZ-infected HUVECs (only 3 μ g of nuclear extract was used). Note the absence of TAL-1/E DNA complex in nuclear extract from ECs infected with Ad-TRE- Δ bas. (C) The effect of wt or Δ bas TAL-1 overexpression on in vitro angiogenesis. Angiogenesis was monitored using adenovirus-infected ECs plated on Matrigel. Representative photographs of HUVECs after 90 minutes and 20 hours of plating are shown.

These in vitro experiments showed that wt TAL-1 ectopic expression accelerates the process of tubule-like morphogenesis, while Δ bas overexpression completely inhibits this process. Therefore, TAL-1 activity appears to function as a signal to stimulate endothelial cell morphogenesis as measured in this in vitro assay.

Effects of wild-type and Δ bas TAL-1 overexpression on EC proliferation and migration

Both proliferation and migration of ECs are essential early events of angiogenesis. Therefore, we investigated whether TAL-1 ectopic overexpression affected either or both of these processes.

To assess the effect of TAL-1 on cell proliferation, the same number of HUVECs were infected with Ad-Tet-Off and with one of the following, TRE-LacZ, TRE-wtTAL-1 or TRE- Δ bas and cultured in complete medium. Cell numbers were counted at 72 hours post-infection. As shown in Fig. 4A, no major differences were observed between the different populations, except for a slight increase in cell numbers (15%) associated with wtTAL-1 overexpression. This indicates that neither wt nor Δ bas TAL-1 overexpression markedly modifies EC proliferation.

Cell migration was assessed with HUVECs and UCB-ECs transduced with the different adenoviruses using Boyden chamber assays. As presented in Fig. 4B, similar results were obtained with HUVECs and UCB-ECs. Migration was stimulated by the chemoattractant medium in control LacZ-infected cells (about 2-fold over background). In contrast, when ECs were transduced with either TRE-wtTAL-1 or TRE- Δ bas, there was no increase in the number of cells transmigrating towards chemoattractant medium as compared with control medium (background). These experiments demonstrate that ectopic overexpression of either wt or Δ bas TAL-1 in ECs inhibits their chemotactic activities towards angiogenic factors as measured in this in vitro assay.

Effects of wild-type and Δ bas TAL-1 overexpression on vascularisation in mice

Based on our in vitro studies, we used the mouse model of subcutaneously implanted Matrigel to evaluate the in vivo effects of TAL-1 (SCID mice). Each animal received three implants containing Ad-Tet-Off together with either Ad-TRE-LacZ, used here as the control, Ad-TRE-wtTAL-1 or Ad-TRE- Δ bas. In this assay, host ECs that infiltrate the plug become transduced and express the transgene. On day 5 or 7, animals were sacrificed and implants were photographed and processed for histological analysis. The experiment was repeated three times with five or six animals, such that a total of 16 implants for each Ad-TRE vector was analyzed.

Macroscopic analysis showed no consistent differences between each of the three experiments. Fig. 5A shows representative experiments derived from two animals. In control Ad-LacZ formulated plugs, there was a significant angiogenic response as indicated by the pink/orange hue throughout the plug. In 50% of the Ad-wtTAL-1 formulated plugs (8/16) angiogenesis was markedly enhanced as judged by the much redder color of the implants; in the other 50%,

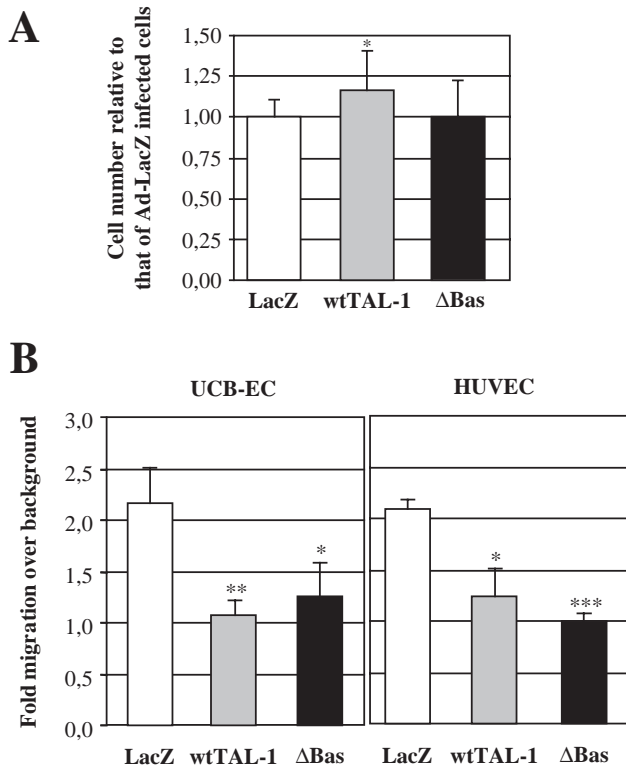


Fig. 4. Effects of wild-type and Δ bas TAL-1 overexpression on cell proliferation and migration. (A) 7×10^4 HUVECs were seeded onto collagen-coated 12-well plates and infected with Ad-Tet-Off and with either Ad-LacZ or Ad-wtTAL-1 or Ad- Δ bas and cultured in complete medium. Cell numbers were monitored at 72 hours post-infection by direct counting. The number of LacZ-infected ECs was arbitrarily set at 100. The values are the mean \pm s.d. of independent infection experiments ($n=10$); statistical significance relative to Ad-LacZ infected cells is indicated: * $P < 0.05$ (Student's t -test). (B) HUVECs or UCB-ECs were co-infected with Ad-Tet-Off and with Ad-TRE-wtTAL-1, Ad-TRE- Δ bas or Ad-TRE-LacZ. Twenty hours post-infection, 4×10^4 cells were placed in the upper wells of 8 μ m cell culture inserts in serum-free medium. The lower wells contained either control medium or chemoattractant medium. After 90 minutes, cells were fixed and labeled with propidium iodide. Migration was quantified by counting the cells that had migrated through the inserts in 6 randomly selected fields (original magnification, $\times 10$). The number of cells that had migrated through the inserts towards a serum-free medium was considered as background and was arbitrarily set at 1. The values are the mean \pm s.d. of three independent infection experiments; statistical significance relative to Ad-LacZ infected cells is indicated: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's t -test).

the angiogenic response appeared similar to that of control Ad-LacZ plugs, and this occurred essentially when vascularization of the control implants was already high. In contrast, a large majority of Ad- Δ -bas formulated implants (15/16) were almost white, indicating a strong reduction of angiogenesis as compared to control plugs.

Histological analysis of the plugs after Hematoxylin and Eosin staining were consistent with the macroscopic observations (Fig. 5B). Importantly, EC infiltration of the plugs, as assessed by CD31 immunostaining, were detected in all plugs (see Fig. 5C). However, the densities and the

organization of ECs were quite different between the three types of formulated implants. In control LacZ implants, histological analysis showed the presence of numerous endothelial cells well organized into tube-like structures; red blood cells appeared in some of these capillaries indicating their functionality (see Fig. 5B). In wtTAL-1 transduced implants, although EC density seemed similar to that of the control, blood vessel morphology was strikingly different: vascular structures with a lumen were more numerous and there was a significant increase in cross-sectional diameter (Fig. 5B,C); indeed, many ECs were already organized either into capillaries or into large vascular cavities engorged with blood cells. A typical vascular lacuna associated with wtTAL-1-transduced implants is shown in Fig. 5B. In contrast, the implants transduced with Ad- Δ bas had only occasional small-caliber capillary-like structures and no red blood cells were present, consistent with the white color of the implant. The large majority of the infiltrated ECs were organized as chains of cells at the host tissue/Matrigel boundary but no lumen formation was visible (Fig. 5B,C).

Altogether, these experiments indicate that wtTAL-1 transduction in ECs enhances in vivo angiogenesis, whereas that of Δ bas mutant markedly blocks this process.

tal-1 silencing in ECs and its effects on in vitro angiogenesis

We next wanted to investigate whether TAL-1 protein was absolutely required for angiogenesis. Small interfering double-stranded RNAs (siRNAs) have become a powerful tool to knockdown specific gene expression in mammalian cells (Gitlin et al., 2002; Yu et al., 2002). Transfections of HUVECs were carried out with siRNA targeting *tal-1* coding sequences; siRNAs targeting irrelevant sequences, not transcribed in ECs, were used as a control. 24 hours after transfection, whole cell lysates were assayed for TAL-1 expression by immunoblotting. Endogenous TAL-1 expression was repeatedly abolished with the *tal-1* siRNA while TAL-1 levels were constant for control siRNA- and mock-treated samples. These experiments, as illustrated in Fig. 6A, demonstrated that endogenous TAL-1 expression was efficiently blocked by transfection of *tal-1* siRNA. We then investigated the effects of *tal-1* knockdown on in vitro endothelial differentiation on Matrigel. Several experiments were carried out with HUVECs. We noticed a significant lag in the appearance of cords in both *tal-1* and control siRNA-transfected ECs (two different control siRNAs were tested) as compared to mock-transfected cells (Fig. 6B), indicating that the entry of siRNAs into primary ECs provokes non-specific adverse biological effects. At 24 hours, we found no obvious differences between the resulting tubule-like networks formed by *tal-1* and control siRNA-transfected ECs (not shown). However, an additional delay in the formation of the cord-like structures was repeatedly observed with *tal-1* siRNA-transfected ECs as compared to control siRNA-transfected ECs (Fig. 6B). Thus, the knockdown of TAL-1 protein, even if it slightly delays it, does not prevent in vitro endothelial cell morphogenesis.

Discussion

In this study, we have investigated the biological function of

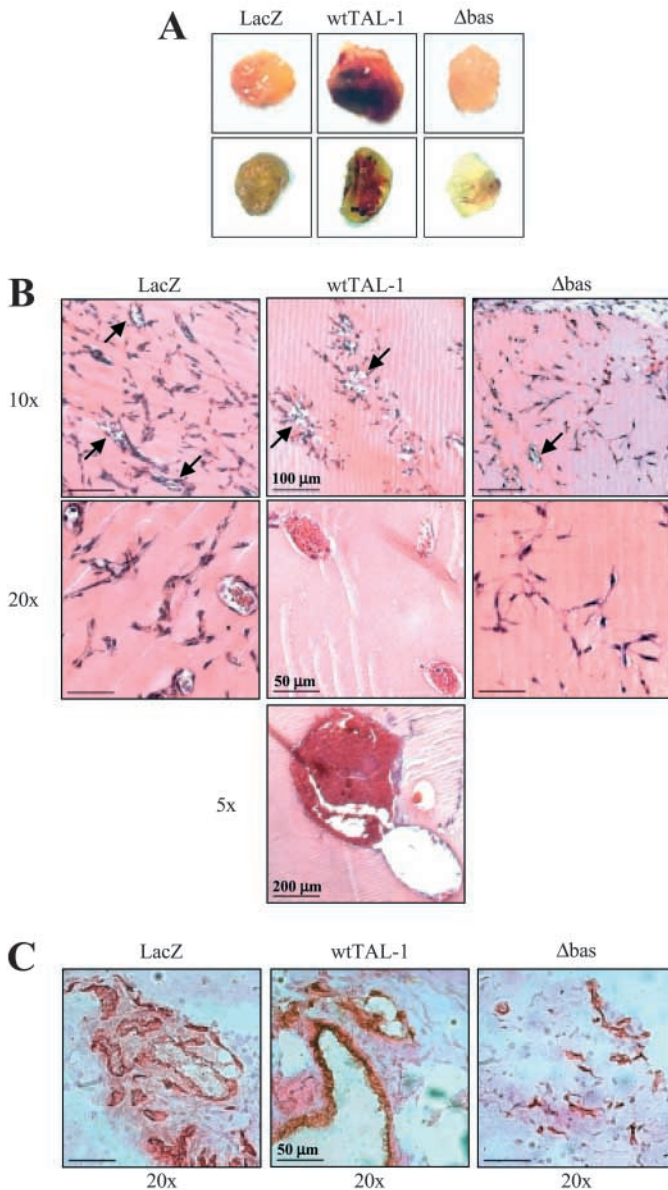


Fig. 5. Effects of wild-type and Δ bas TAL-1 ectopic expression on in vivo angiogenesis. Male SCID mice were injected subcutaneously at three sites with 0.5 ml Matrigel mixed with bFGF, heparin and Ad-Tet-Off together with each Ad-TRE. Injection sites were chosen such that each animal received the three Ad-TRE-LacZ, wtTAL-1 and Δ bas. Three experiments were carried out with five or six animals. Matrigel plugs were removed after 6 days, photographed and processed for histology. *Macroscopic observations.* Representative photographs of three Matrigel implants formulated with the indicated adenovirus harvested from two individual mice belonging to two different experimental groups. Note the increase in angiogenesis (as demonstrated by brown/red color) in wt-TAL-1-transduced implants and its inhibition in Δ -bas-transduced implants (almost white) as compared to control LacZ implants. *Histological analyses.* (Top) Representative images of Matrigel plug sections after Hematoxylin and Eosin staining; capillary structures with lumen (indicated by arrows) were more numerous in wtTAL-1 and very rare in Δ bas as compared to control implants (LacZ). Higher magnification ($\times 20$) shows the presence of red blood cells within these structures only in wtTAL-1- and LacZ- but not in Δ bas-formulated implants. (Bottom) Low magnification image ($\times 5$) illustrating the very large vascular structures with large vascular cavities engorged with red blood cells observed in wt-TAL-1-transduced implants. (C) Immunohistochemical staining with anti-CD31 antibody. Representative photographs illustrating the infiltration of CD31-positive ECs (appearing in brown) and their different organization within the three types of Matrigel implants.

TAL-1 in angiogenesis. We have assessed the effects of constitutive expression of wt or a dominant-negative mutant (Δ bas) TAL-1 on angiogenesis. Our in vitro analysis relied on two angiogenic assays: chemotactic migration of endothelial cells and their differentiation into capillary-like structures on Matrigel. The in vivo effects of TAL-1 were investigated on vascularisation occurring in Matrigel subcutaneously implanted in mice. With regard to the key steps in the angiogenic process, our current studies have identified TAL-1 as a positive factor for cell morphogenesis and as a regulator of cell motility.

Endothelial cells from different sources are known to be heterogeneous; they differ in their expression of adhesion proteins, and responsiveness to cytokines (Garlanda and Dejana, 1997; Mason et al., 1997; Salcedo et al., 2000). Our study now shows that these distinct endothelial cells also differ with regard to TAL-1 expression. While TAL-1 proteins were detected in all types of proliferating ECs tested, their levels ranged from nearly undetectable in ECs derived from bone

marrow to high in ECs derived from microvessels. When proliferating ECs were brought to quiescence and deprived of endothelial growth factors, a progressive disappearance of TAL-1 protein occurred and correlated with a rapid down-regulation of *tal-1* mRNA levels. As the half-life of the TAL-1 protein is 4 hours (C.D., unpublished), its degradation was not significantly accelerated by starvation conditions. The lack of EC-activating factors was directly responsible for *tal-1* down-regulation since quiescent ECs maintained in a medium containing these growth factors still expressed TAL-1. These findings are consistent with the *tal-1* expression profile in vivo: TAL-1 protein is not detected in resting vasculature deprived of pro-angiogenic factors but is expressed in newly forming vessels in which ECs are under the influence of angiogenic stimuli (Chetty et al., 1997; Hwang et al., 1993; Kallianpur et al., 1994).

Our studies show that TAL-1 protein expression is modulated during the process of in vitro angiogenesis and significantly, this was observed with ECs derived from three different tissues (large vessels, small vessels and blood circulating endothelial precursors). Proliferative and migratory activities of ECs constitute basal mechanisms of in vivo angiogenesis. Several observations indicate that TAL-1 does not directly modulate endothelial cell proliferation. First, quiescent ECs, when maintained in the presence of endothelial growth factors, expressed TAL-1 at even higher levels than proliferating cells (about 2-fold, data not shown). Second, our data show that ectopic overexpression of wt or Δ bas TAL-1 did not modify endothelial cell growth. Finally, in *tal-1*-deficient embryos, no reduction in the number of endothelial cells was observed (Visvader et al., 1998).

A significant reduction in TAL-1 protein level occurs during the first hours of in vitro angiogenesis at the time when the cells are forming chains. This TAL-1 decrease was high in

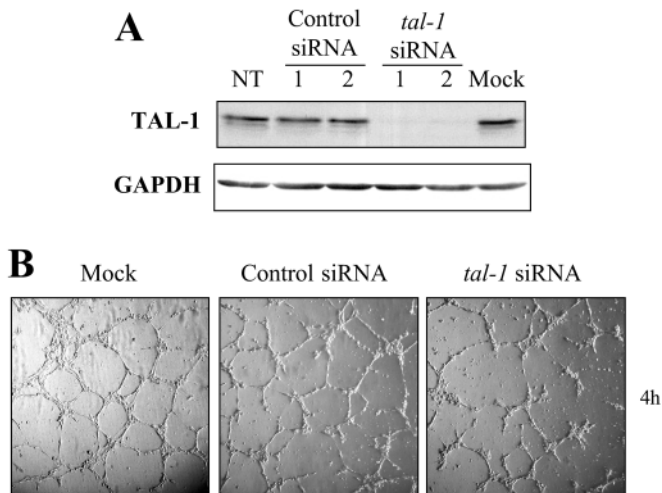


Fig. 6. Effects of TAL-1 knockdown on in vitro angiogenesis. HUVECs were transfected with *tal-1* or control siRNA and analyzed 24 hours after the second transfection for TAL-1 protein content or in vitro angiogenesis. *tal-1* silencing in endothelial cells by RNA interference. TAL-1 expression was assessed in whole cell lysates (30 μ g) by immunoblotting with the anti-TAL-1 mAb 3BTL73. The blot was reprobed with an anti-GAPDH rabbit antiserum to control protein loading. Effect of TAL-1 knockdown on in vitro angiogenesis. Representative photographs of transfected HUVECs after 4 hours of plating on Matrigel.

microvascular HMEC-1 cells and in UCB-ECs (80% and 60% reduction, respectively) but rather modest in HUVECs (20% reduction). If we consider the 4-hour half-life of TAL-1 protein, TAL-1 degradation is accelerated in both HMEC-1 and UCB-ECs but not in HUVECs. These observations are consistent with a recent report demonstrating that hypoxia, a well-known angiogenic signal, induces accelerated turnover of TAL-1 in proliferating microvascular ECs but not in ECs from large vessels (HUVECs) (Tang et al., 2002). Hypoxia-induced acceleration of TAL-1 degradation was shown to be mediated by mitogen-associated protein kinase (MAP kinase) through phosphorylation of Ser¹²² that serves as a signal for ubiquitination and degradation by the proteasome (Tang et al., 2002). The decrease in TAL-1 during the early steps of angiogenesis is likely to be also mediated through MAP kinase phosphorylation.

Our data show that TAL-1 activity modulates motility properties of endothelial cells. Cell migration is a complex dynamic process, which includes directed motion requiring cell polarization and random walk motion. As we discussed above, down-expression of endogenous TAL-1 happens during in vitro angiogenesis at the time when the cells are moving in all directions to form chains. In addition, our in vitro assays show that TAL-1 overexpression inhibits migration in the direction of chemoattractant signals. The assays used in this study (Boyden assays) do not provide information about the dynamics of cell migration or the mode of motility, adhesion-based or amoeboid (Sahai and Marshall, 2003). Thus, it is possible that the apparent inhibition of chemotactic migration associated with TAL-1 overexpression reflects a change in the mode of cell motility. Time-lapse analysis of cell movement as well as cell behavior in a 3D environment will be required to delineate how TAL-1 regulates endothelial cell motility.

Our findings clearly link TAL-1 activity to endothelial morphogenesis. This is consistent with the high levels of TAL-1 expression in forming vessels (Chetty et al., 1997; Kallianpur et al., 1994). First, the highest expression of endogenous TAL-1 during in vitro angiogenesis corresponded to the window of capillary-like formation. Second, we found that ectopic wtTAL-1 accelerated the appearance of those cytoplasmic extensions that precede the formation of cords in vitro. In agreement with this, the marked increase in angiogenesis observed in Matrigel implants transduced with wild-type TAL-1 was associated with a general enlargement of capillary lumens, which in some cases led to the formation of large vascular cavities. Finally, the dominant-negative mutant Δ bas completely impaired all these processes.

tal-1 inactivation by RNA interference did not impede capillary-like network formation in vitro but only delayed the onset of tubule-like differentiation, indicating that TAL-1 is not absolutely required for EC morphogenesis, at least in vitro. This finding was not expected from the phenotype of *tal-1*^{-/-} embryos rescued for *tal-1* expression in hematopoietic tissues; these embryos died by day 9.5 p.c. (E9.5) because of severe defects in yolk sac vascular remodeling (Visvader et al., 1998). Nevertheless, these latter observations are not inconsistent with those reported here, since our experiments involved angiogenesis of ECs derived from adult tissues rather than from early embryos. One possibility is that another factor expressed in mature ECs, but not in embryonic endothelial cell lineage at E9.5, may compensate the lack of TAL-1 activity in the process of cell morphogenesis. This possibility is supported by our findings that the dominant-negative Δ bas strongly inhibits angiogenesis. Indeed, the Δ bas mutant, although lacking a DNA-binding domain, is still able to mediate protein interactions through its HLH domain. Hence, one of the consequences of Δ bas overexpression could be not only the competition with TAL-1 for its partners, but also that of other HLH-interacting proteins; the function of some of these proteins might be redundant with TAL-1, at least for mediating endothelial morphogenesis.

To our knowledge, no partners of TAL-1 have been identified in endothelial cells. We show here that the bHLH E47 heterodimerizes with TAL-1 to bind specific E-box sequences in endothelial cells, similar to what has been described in hematopoietic cells (Hsu et al., 1994a; Hsu et al., 1994b). In immature erythroid cells, TAL-1 is part of a large multifactorial nuclear complex, which includes E47, GATA-1, LMO-2 and its regulator Ldb1 (Wadman et al., 1997) and transactivates the *c-kit* gene (Kros1 et al., 1998; Vitelli et al., 2000). LMO-2 was found to be required for proper vascular network formation during developmental angiogenesis (Yamada et al., 2000) and has recently been proposed to be a key regulator of tumor angiogenesis (Yamada et al., 2002). Because LMO-2 and TAL-1 are able to directly interact, it is tempting to speculate that both proteins may be present in the same multiprotein complexes in endothelial cells to mediate specific cellular events during angiogenesis. Like myogenic and neural bHLH proteins (reviewed by Massari and Murre, 2000), endothelial-specific bHLH factors may control the transcription of a set of genes according to a highly regulated hierarchy. We propose that the activity of multiprotein complexes including TAL-1 and LMO-2 is modulated by the presence or the absence of certain HLH proteins. In this model, distinct events during

angiogenesis might be regulated by the expression of one specific HLH protein. Notably, the observation that Δ bas-overexpression produces the same inhibitory effects on chemotactic cell migration as does that of wtTAL-1 strongly suggests that endothelial migration might be mediated by an unknown HLH-containing or HLH-interacting protein.

Id proteins, which lack a basic domain, have been proposed to modulate the timing of bHLH activity during angiogenesis (Lyden et al., 1999). The absence of Id1/Id3 in mice embryos leads to severe defects in the secondary vascular network, and a partial reduction in Id results in angiogenic defects in adult mice (Lyden et al., 2001; Lyden et al., 1999). Significantly, the expression profile of Id1/Id3 parallels that of TAL-1 (Chetty et al., 1997; Kallianpur et al., 1994): both are detected at high levels in the vasculature of human tumors, in newly formed vessels but not in resting vasculature. Although Id proteins do not interact directly with TAL-1, they efficiently dimerize with the TAL-1 partner E47, and as a consequence, inhibit the formation of TAL-1/E47 heterodimers. Therefore, TAL-1 may be one of the bHLH factors that Id proteins regulate in endothelial cells.

While our experiments clearly identify TAL-1 as a regulator of endothelial cell morphogenesis, the mechanisms by which it does so remain to be determined. Certain clues, however, can be derived from our observations. Non confluent control ECs (i.e. infected with Ad-TRE-LacZ) plated in complete endothelial-specific medium displayed a classic elongated spindle-shaped morphology. In contrast, most TAL-1 overexpressing ECs displayed a more spread out and flattened morphology (data not shown). We propose that TAL-1 mediates morphological changes in endothelial cells, which allow their differentiation into cords and tubules. Further studies will aim to identify the signaling pathways downstream of TAL-1 that mediate cytoskeleton rearrangements required for proper endothelial morphogenic differentiation.

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