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## Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1☆

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### Abstract

Angiogenesis is a multistep process of critical importance both in development and in physiological and pathophysiological processes in the adult. It involves endothelial cell (EC) sprouting from the parent vessel, followed by migration, proliferation, alignment, tube formation, and anastomosis to other vessels. Several in vitro models have attempted to recreate this complex sequence of events with varying degrees of success. We report an optimized protocol for human umbilical vein EC in which EC sprout from the surface of beads embedded in fibrin gels. Fibroblast-derived factors, other than Angiopoietin-1, promote sprouting, lumen formation, and long-term stability of neovessels. Analysis by time-lapse and still photomicroscopy demonstrates dynamic vessels guided by a “tip cell” that extends numerous processes into the gel. Behind this cell a lumen forms, surrounded by a single layer of polarized EC. The growing sprouts express *notch 1*, *notch 4*, and *delta 4*, as well as the downstream notch effector *HESR-1*. Importantly, cells can be infected with adenovirus to high efficiency without compromising sprout formation, thus allowing for manipulation of gene expression. This improved model recapitulates all the major steps of angiogenesis seen in vivo and provides a powerful model for analysis of this complex phenomenon.

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### Introduction

All cellular tissues are critically dependent on an adequate blood supply and it is through the process of

angiogenesis—the growth of new vessels from preexisting vessels—that this is accomplished (Carmeliet, 2000; Folkman, 1975; Folkman, 1985; Risau, 1997). As a consequence of this dependence, angiogenesis is crucial to development of the embryo as well as to several physiologic and pathologic processes: both the female reproductive cycle and wound healing are critically dependent on angiogenesis, as is the growth of solid tumors (Conway et al., 2001).

Numerous in vivo models for studying angiogenesis have been developed, including injection of Matrigel or implantable tumors into mice, the corneal pocket assay, and the chick CAM assay (Akhtar et al., 2002; Folkman, 1975; Muthukkaruppan et al., 1982). All have their strengths and weaknesses, most notably the difficulty in manipulating

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gene expression in the endothelial cells (EC), and the difficulty in purifying angiogenic EC for biochemical and gene expression analyses. For this reason a number of *in vitro* assays have been developed (Auerbach et al., 2003). Although these may often model some aspects of angiogenesis, for example, EC migration or proliferation assays using collagen gels or Matrigel fail to model sprouting. In addition, if lumens form at all, they are often slitlike and intracellular rather than surrounded by multiple, polarized EC. These assays do offer the advantage of being relatively quick, the cells can be transfected, and pure populations of EC can be recovered. A number of reports have shown that mesenchymal cells such as fibroblasts can provide important factors that modulate growth and stability of capillary sprouts in collagen gels (Montesano et al., 1993; Tille and Pepper, 2002; Velazquez et al., 2002; Villaschi and Nicosia, 1994), although in one case this ability was limited to a single line of fibroblasts (Montesano et al., 1993). Other types of mesenchymal cells such as smooth muscle cells (SMC) may also act in a similar fashion. SMC inhibit EC proliferation and new vessel formation in cocultures, implying that mural cells may interact with EC to stabilize newly formed capillaries (Darland and D'Amore, 2001; Orledge and D'Amore, 1987). The aortic ring assay and its variants provide excellent recapitulation of the various stages of angiogenesis (Nicosia and Ottinetti, 1990); however, the cultures contain multiple cell types and reliable transfection is problematic.

Recently a new system was developed, which involves culturing EC as a monolayer on Cytodex beads and then embedding the beads in fibrin gels (Nehls and Drenckhahn, 1995a, 1995b). Bovine aortic EC (BAEC) have been shown to bud and form capillary-like sprouts in response to growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). This system has been well characterized and represents a major advance in *in vitro* angiogenesis assays. A drawback, however, is that the published technique does not support sprouting of human umbilical vein EC (HUVEC). These cells have for many years been the canonical EC model system and a vast literature exists on their physiology and gene expression profiles under numerous conditions. For this reason we have optimized the method for HUVEC and can now reproducibly induce HUVEC to form multicellular capillaries in fibrin gels. We have found that skin fibroblasts (SF) growing on top of the gels provide important signals that lead to maturation of the capillaries. The EC sprout, divide, migrate, branch, form lumens, and anastomose to form networks in much the same way as capillaries *in vivo* (Folkman, 1985). The cells express *HESR-1*, *notch 1*, *notch 4*, and *delta 4*, genes characteristic of angiogenic EC (Henderson et al., 2001; Mailhos et al., 2001; Taylor et al., 2002) and can be infected with adenovirus and harvested for further analysis.

## Materials and methods

### Cell culture

HUVEC were isolated from umbilical cords obtained through local hospitals under approval of the appropriate IRBs. HUVEC were routinely grown in M199 (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) (Gibco) and endothelial cell growth supplement (ECGS) (BD Biosciences, Bedford, MA) at 37°C and 5% CO<sub>2</sub>. HUVEC between P3 and P4 were used for all experiments. Lung microvascular EC (BioWhittaker, Walkersville, MD) were routinely grown in EGM-2 (BioWhittaker, Walkersville, MD) at 37°C and 5% CO<sub>2</sub>. HMVEC-L between P6 and P8 were used for all experiments. Bovine aortic EC (BAEC) (BioWhittaker, Walkersville, MD) were routinely grown in EGM-MV (BioWhittaker, Walkersville, MD) at 37°C and 5% CO<sub>2</sub>. BAEC between P3 and P4 were used for all experiments. SF (Detroit 551) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). SF were routinely grown in M199 supplemented with 10% FBS at 37°C and 5% CO<sub>2</sub>. Skin fibroblasts (SF) between P2 and P20 were used for all experiments. For collection of conditioned media (CM), SF were grown to 25% confluency in M199 supplemented with 10% FBS. Medium was switched to EGM-2 and SF were then grown to 90% confluency. Medium was collected and frozen.

### Fibrin bead assay

HUVEC were mixed with dextran-coated Cytodex 3 microcarriers (Amersham Pharmacia Biotech, Piscataway, NJ) at a concentration of 400 HUVEC per bead in 1 ml of EGM-2 medium (Clonetics, Walkersville, MD). Beads with cells were shaken gently every 20 min for 4 h at 37°C and 5% CO<sub>2</sub>. After incubating, beads with cells were transferred to a 25-cm<sup>2</sup> tissue culture flask (BD Biosciences, Bedford, MA) and left for 12–16 h in 5 ml of EGM-2 at 37°C and 5% CO<sub>2</sub>. The following day, beads with cells were washed three times with 1 ml of EGM-2 and resuspended at a concentration of 200 cell-coated beads/ml in 2.5 mg/ml of fibrinogen (Sigma, St. Louis, MO) with or without 0.15 units/ml of aprotinin (Sigma) at a pH of 7.4. Five hundred microliters of fibrinogen/bead solution was added to 0.625 units of thrombin (Sigma) in one well of a 24-well tissue culture plate. Fibrinogen/bead solution was allowed to clot for 5 min at room temperature and then at 37°C and 5% CO<sub>2</sub> for 20 min. One milliliter of EGM-2 (which contains 2% FBS) with or without 0.15 units/ml aprotinin was added to each well and equilibrated with the fibrin clot for 30 min at 37°C and 5% CO<sub>2</sub>. Medium was removed from the well and replaced with 1 ml of fresh medium with or without 0.15 units/ml aprotinin or additional growth factors. Twenty thousand SF were plated on top of the clot and medium was changed every other day. Bead assays were monitored for 7 days. VEGF<sub>165</sub>, bFGF, Angiopoietin-1 (Ang-1), and transforming

growth factor- $\beta$  (TGF- $\beta$ ) (R&D Systems, Minneapolis, MN) were used at the indicated concentrations. In these experiments the VEGF that is normally part of the EGM-2 formulation was omitted. A detailed protocol is available on request.

#### *Collagen gel assay*

The collagen gel assay was previously described (Henderson et al., 2001). Wells of a six-well tissue culture plate were coated with 1.5 mg/ml of rat tail collagen type I and 3  $\mu$ g/ml of fibronectin (BD Biosciences, Bedford, MA). HUVEC ( $5 \times 10^5$ ) were seeded in each collagen-coated well with M199, 20% FBS, and ECGS and allowed to attach for 1 h. A second collagen layer was added to a well and allowed to polymerize. HUVEC were then fed with M199 supplemented with 10% FBS and 25 ng/ml VEGF and bFGF.

#### *Quantitation of vessels in vitro*

High-resolution images of beads were captured on an IX70 Olympus microscope with a 4X objective. Capturing images at low magnification has the advantage of a high depth of field, which enables inclusion of all sprouts in focus. Images were then magnified in Adobe Photoshop (Adobe Systems Inc., San Jose, CA) and analyzed in NIH ImageJ. Number of sprouts per bead was determined, where a sprout is defined as a vessel of length equal to the diameter of a bead. Sprout length was also measured in arbitrary units. For statistical analysis, 25 beads were assessed for each condition.

#### *Time-lapse video microscopy*

HUVEC-coated beads were cultured in fibrin gels for 2–3 days and then transferred to the stage of a Nikon Eclipse TE300, equipped with multidimensional  $x$ ,  $y$ , and  $z$  axes and maintained at 37°C and 5% CO<sub>2</sub> for 72 h. Images were captured from multiple beads every 20 min using Metamorph software (Universal Imaging Corporation, Downingtown PA) and saved as TIFF files. Individual files were then combined and processed into QuickTime movies (Apple, Cupertino, CA) using Metamorph software.

#### *Adenoviral infection of cells*

Recombinant adenoviral plasmid expressing green fluorescent protein (GFP) (Ad-GFP) was kindly provided by Dr. Luis Villarreal (UCI, Viral Vector Facility). Additionally, the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) was also used to generate Ad-GFP. Adenoviral plasmids were purified, linearized, and transfected into HEK-293 cells (ATCC, Rockville, MD) for production of high-titer adenoviral stocks. Adenoviral titer was determined using the “spot assay” as previously described (Be-

wig and Schmidt, 2000). Ad-GFP were aliquoted and stored at  $-80^\circ\text{C}$  until further use. Ad-GFP were added to HUVEC (70–80% confluent) at multiplicity of infection (MOIs) from 1 to 1000. Infections of HUVEC were carried out in EGM-2 at 37°C and 5% CO<sub>2</sub> for 24 h. Following this period, cells were harvested and analyzed by flow cytometry or cultured in bead assays.

#### *RT-PCR analysis*

Fibrin gels were treated with 10X trypsin-EDTA (Gibco) for 2–3 min to remove the top layer of SF. An additional 1 ml of 10X trypsin-EDTA was added to the fibrin gel to release the HUVEC-coated beads. HUVEC-coated beads were then washed with M199 supplemented with 40% FBS and centrifuged to disrupt the beads and release the HUVEC. This protocol routinely resulted in recovery of cells that were greater than 98% CD31 positive by FACS (data not shown). Total RNA was isolated from HUVEC and SF grown as a monolayer using TRIzol (Invitrogen, San Diego, CA). HUVEC on beads were isolated as described above and total RNA was recovered using TRIzol. Following RQ1 DNase treatment (Fisher Scientific, Pittsburg, PA), cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) was performed using *Taq* polymerase (Invitrogen) for 30 cycles at annealing temperatures of 60.5°C for *Tie-2*, 66°C for *notch 1* and *notch 4*, 62°C for *delta 4*, and 63.5°C for *HESR-1*. The primer sequences and expected fragment sizes were as follows: *Tie-2* upper 5'-GGAAGCATGGACTCTTTAGC-3' and lower 5'-CACAGAAATAAGCACCATTGA-3' (315 bp), *notch1* upper 5'-GCGGCCGCCTTTGTGCTTCTGTTC-3' and lower 5'-CGGCCGGCGCGTCCTCCTT-3' (538 bp), *notch4* upper 5'-TCCTGGGGCCCCGGGCTGAAGAAAAG-3' and lower 5'-ACGCCGATGAGCTGGAGGACGAGA-3' (394 bp), *delta4* upper 5'-GCCGGGTACCTTCTCGCTCATCATC-3' and lower 5'-GCCTCCCCAGCCCTCATCACAAGTA-3' (502 bp), *HESR-1* upper 5'-GGAGAGGCGCCGCTGTAGTTA-3' and lower 5'-CAAGGGCGTGC GCGTCAAAGTA-3' (429 bp), *GAPDH* upper 5'-ACCACAGTCCATGCCATCAC-3' and lower 5'-TCCACCACCCTGTTGCTGTA-3' (450 bp).

#### *Histology and immunostaining*

For EC nuclei staining, fibrin gel clots were washed twice with 1X PBS and then fixed overnight in 2% paraformaldehyde. Fibrin gel clots were washed twice again with 1X PBS and then stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma). Fluorescence was monitored using an IX70 Olympus microscope with an IX-FLA fluorescence attachment. For immunoperoxidase methods, a fibrin bead assay was performed as above with the exception that the fibrin clots were formed in transwells to allow easy removal for processing. Membranes and clots were removed, fixed in 10% neutral buffered formalin, and paraffin-embedded. Six-micrometer-thick sections

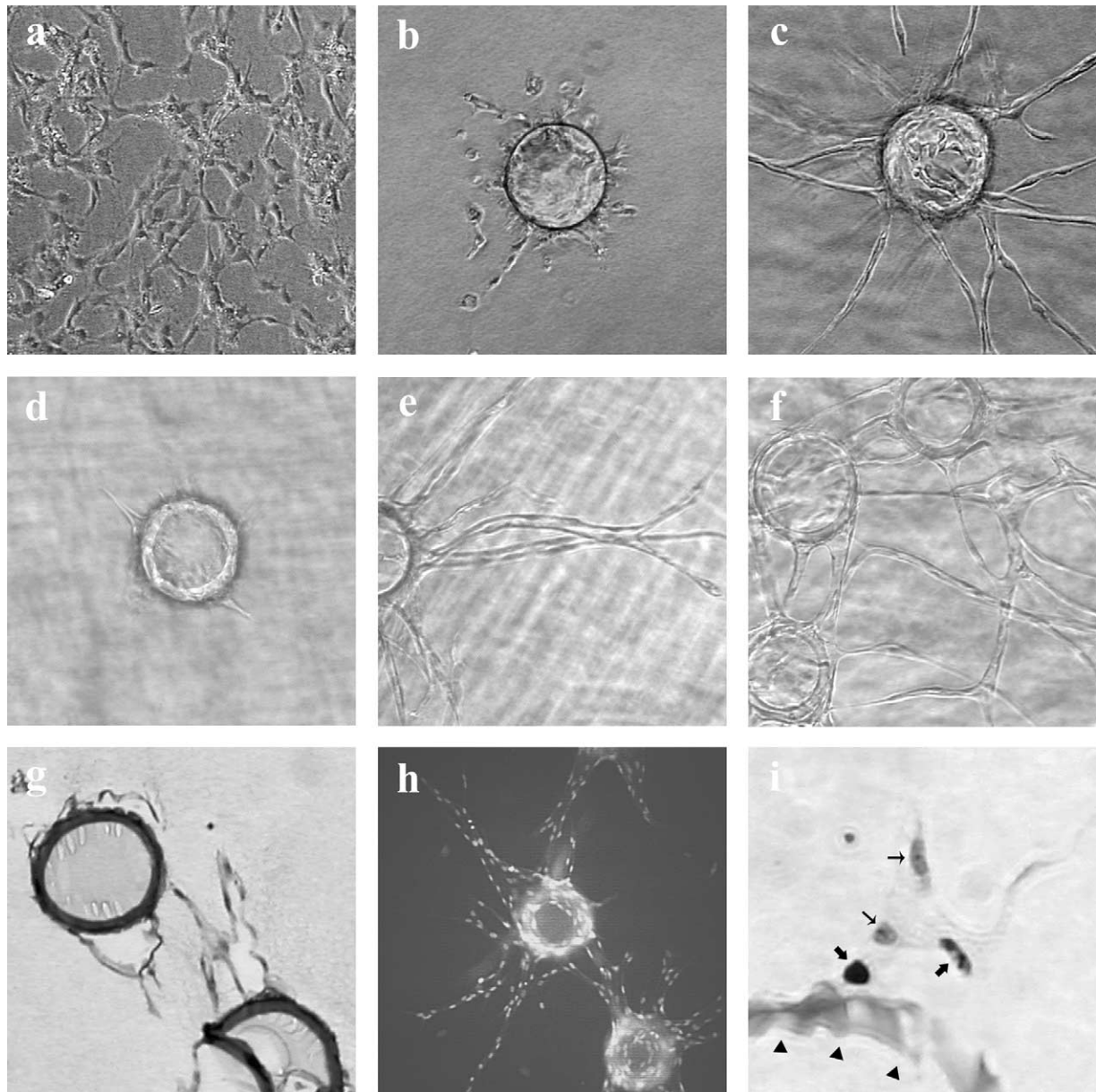


Fig. 1. HUVEC form capillary-like sprouts in fibrin gels. (a) HUVEC grown in collagen gels align to form a primitive vascular plexus. (b) HUVEC-coated bead in the absence of SF. At day 5, the vessels regress into single unattached HUVEC and numerous HUVEC migrate away from the bead. (c) A HUVEC-coated bead in the presence of SF. At day 5, many thin vessels form with distinct lumens. (d) In the presence of SF, the first sprouts appear at day 2. (e) The newly formed sprouts continue to extend from the bead and branch; day 5. (f) At day 7, anastomosis occurs between neighboring vessels forming a complex network. (g) Hematoxylin-and-eosin-stained sections of newly formed vessels demonstrate that tubes are multicellular. (h) DAPI staining of nuclei confirms that sprouts are multicellular and that cells surround the lumen. (i) Staining with a monoclonal Ki-67 antibody reveals the presence of proliferating EC in the newly formed vessels (thick black arrows). Nondividing cells counterstained with hematoxylin are highlighted with thin black arrows. Black arrowheads indicate the surface of the bead.

were cut and then stained with Ki-67 monoclonal antibody (DAKO, Carpinteria, CA) and counterstained with hematoxylin (DAKO).

## Results

HUVEC growing in collagen gels can be induced to align into networks (Fig. 1a); however, these vessels tend to

be thin and immature and develop only slitlike intracellular lumens. Fibrin has also been shown to induce EC tube formation (Chalupowicz et al., 1995; Dejana et al., 1987) and so we tested whether HUVEC growing on dextran-coated beads in fibrin gels (Nehls and Herrmann, 1996) would produce more mature capillaries. After 2–3 days HUVEC began to sprout from the beads, forming short, narrow cordlike structures. However, patent lumens were

not observed. Many single cells migrated away from the beads and after 4–5 days the cords tended to disintegrate into disorganized groups of cells or single, highly migratory cells (Fig. 1b). Mesenchymal cells/fibroblasts have been shown to stabilize *in vitro* vascular networks in some systems (Montesano et al., 1993; Tille and Pepper, 2002; Villaschi and Nicosia, 1994). When SF were plated on top of fibrin gels, we noted a dramatic effect on HUVEC morphology (Fig. 1c). Sprouts were apparent by 2 days (Fig. 1d) and these continued to extend for several days (Fig. 1e), often reaching lengths in excess of 4 bead diameters (600–700  $\mu\text{m}$ ). Only very rarely were single cells seen migrating away from the bead, not in contact with other cells. Time-lapse microscopy revealed that the tip cell of each sprout was highly motile—extending numerous fine processes out into the gel (movies are available as supplementary data). These cellular processes were dynamic, often retracting or changing direction. In this respect their behavior was reminiscent of neural growth cone extension. Lumens formed behind the lead cell, as previously described *in vivo* (Folkman, 1984) and appeared to be surrounded by cells as opposed to being the result of coalescing intracellular vacuoles (Egginton and Gerritsen, 2003; Lubarsky and Krasnow, 2003). Branching is a recapitulation of sprouting from the bead, the surface of which can be thought of as a segment of a vessel wall. Branching was observed, whereby cells extended processes, which grew until they made contact with another vessel. At, or soon after, the point of anastomosis, both vessels often appeared to thicken, although this was often transitory. After several days, a complex web of interconnected capillaries was apparent (Fig. 1f).

Although we have optimized this method for HUVEC, we also tested whether it supported capillary-like growth of microvascular and arterial EC. Fibroblasts have been reported to have no effect on microvascular EC morphogenesis in fibrin gels (Nehls et al., 1994); however, we found that SF supported formation of morphologically distinct capillaries from lung microvascular EC grown under identical conditions to HUVEC (data not shown). In the absence of SF, sprouts were disorganized. BAEC did not form distinct vessels, either in the presence or absence of SF, although vigorous sprouting was observed between days 1 and 3 (data not shown).

Histological analysis confirmed that the capillaries are multicellular and that the lumens are surrounded by multiple cells (Fig. 1g). The multicellularity of the vessels was also observed by DAPI staining, which highlights cell nuclei (Fig. 1h). The increase in vessel length is apparently supported by cell proliferation as numerous cells on the beads and within the sprouts stained positive for nuclear Ki-67 antigen, a marker for proliferating cells (Fig. 1i, thick black arrows). Nondividing cells were also present within the vessels (Fig. 1i, thin black arrows). Thus, the addition of SF to the cultures resulted in the formation of a complex network of interconnected vessels. The development of these vessels recapitulates many of the steps known to be essen-

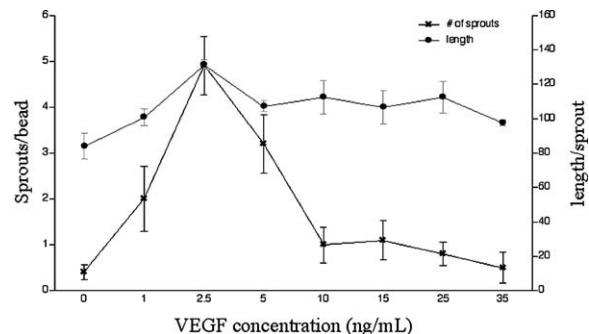


Fig. 2. Sprouting is maximal at low concentrations of VEGF. Bead cultures were established at different concentrations of VEGF and photographed at day 5. Sprout formation is highest at 2.5 ng/ml and decreases as VEGF concentration increases. Average sprout length is highest at 2.5 ng/ml and slightly decreases as VEGF concentration increases. Values represent mean  $\pm$  SEM.

tial for new vessel growth *in vivo*, namely budding, cell migration, cell proliferation, lumen formation, branching, and anastomosis (Folkman, 1985).

We next tested the requirement for bFGF and VEGF. Dose–response experiments revealed that in the presence of SF, exogenously added bFGF was not an absolute requirement for sprouting and vessel growth. There was only a minimal increase in the number of sprouts when bFGF was added and this effect peaked at 2.5 ng/ml (data not shown). Given that in the absence of SF a combination of bFGF and VEGF is required for sprouting and that SF are an important source of bFGF during wound-induced angiogenesis *in vivo*, this finding suggests that SF in these cultures make sufficient bFGF to support robust vessel formation. In contrast, however, in the absence of exogenous VEGF there was very little sprouting (Fig. 2). Consistent with this, SF cultured on fibrin did not produce detectable levels of VEGF (data not shown).

Addition of exogenous VEGF resulted in a biphasic response. Sprout formation was optimal at low concentrations of VEGF (2.5–5 ng/ml) and decreased at higher concentrations (15–35 ng/ml). Correlating with the increase of sprouting, the length of sprouts also tended to increase slightly at low concentrations of VEGF (2.5–5 ng/ml) and decreased at high concentrations of VEGF (15–35 ng/ml) (Fig. 2). We also noted an increase in vessel diameter at higher concentrations of VEGF (Nakatsu et al., submitted). Interestingly, VEGF may not be required for maintenance of the capillaries once they have formed. After 7 days of culture, failure to add fresh VEGF for a further 5 days did not result in regression or disaggregation of the vessels. On the contrary, vessels in most cases continued to exhibit well-developed and patent lumens (Fig. 3h).

We next tested whether the effects of SF could be accounted for by the known proangiogenic factors TGF- $\beta$  and Ang-1 (Koblizek et al., 1998; Madri et al., 1988; Papapetrooulos et al., 1999; Pepper, 1997). Cultures were established in the presence or absence of SF and then cultured in

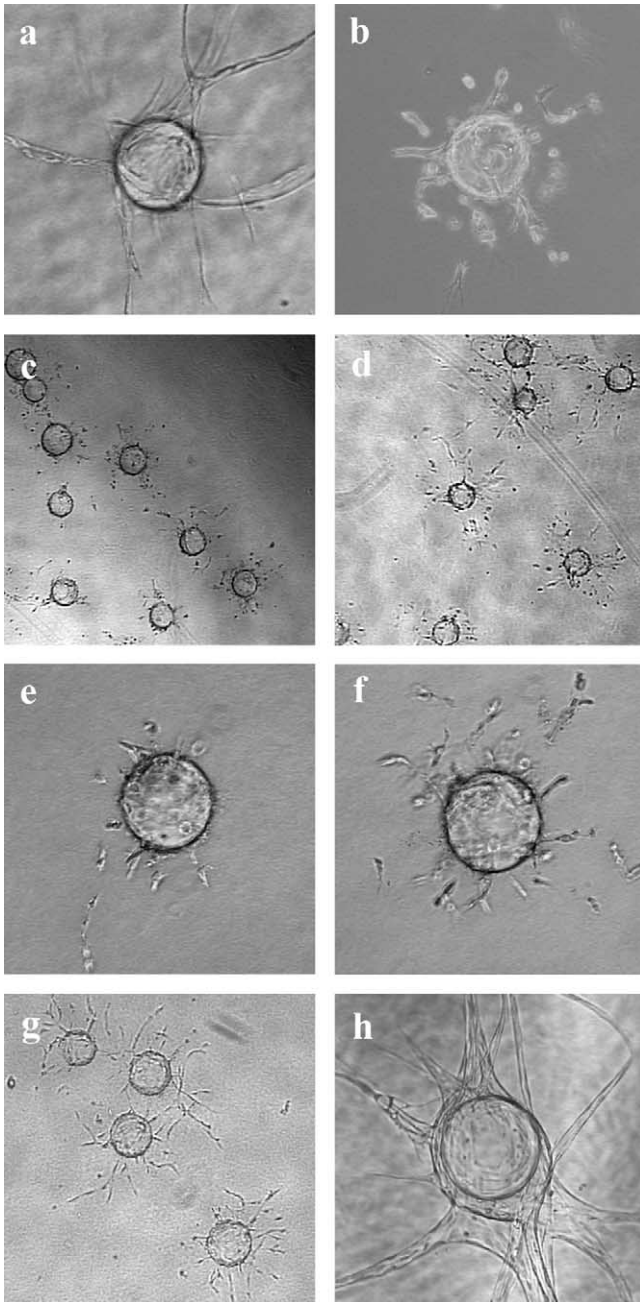


Fig. 3. Neither TGF- $\beta$  nor Ang-1 can completely replace the need for SF. (a) In the presence of SF at day 5, distinct vessels are observed with clear patent lumens. (b) In the absence of SF at day 5, EC migrate away from the bead and vessel formation is limited. (c) TGF- $\beta$  (0.5 ng/ml) shows no discernable effect on sprout formation at day 5 compared to control. Vessel morphology is similar to cultures without SF. (d) TGF- $\beta$  (2 ng/ml) again had no effect on sprouting at day 5. (e) Addition of 1 ng/ml of Ang-1 could not maintain vessel stability at day 5. Vessels regress and numerous migrating EC surround the bead. (f) Even at higher concentrations of Ang-1 (125 ng/ml), vessels are not stabilized at day 5. (g) Conditioned media supports sprouting of thin vessel at day 5; however, no lumen formation is observed. (h) HUVEC-coated bead at day 12, grown for the final 5 days in the absence of fresh VEGF. Vessels are thick and mature with well-defined lumens.

various concentrations of recombinant growth factor for up to 7 days. Compared to HUVEC cultured in the presence of SF (Fig. 3a), HUVEC grown without SF sprouted poorly

and formed few morphologically mature capillaries (Fig. 3b). Neither TGF- $\beta$  nor Ang-1 were able to maintain long-term stability of the vascular networks (Fig. 3c–f). TGF- $\beta$  has consistently failed to promote angiogenesis in vitro, although it is effective in vivo (Pepper, 1997). More surprising, perhaps, was the failure of Ang-1. At early times (1–3 days), Ang-1 promoted increased sprouting and this effect was synergistic with SF (data not shown). Significantly, however, the vessels lost their integrity after several days and disaggregated into sheets of cells surrounding the beads or into single, migratory cells (Fig. 3e and f). Thus, neither TGF- $\beta$ , nor Ang-1 completely replaces the requirement for SF in these cultures.

To pursue the contribution of SF further we prepared conditioned medium (CM) and added this to HUVEC-coated beads embedded in fibrin gels. Consistent with production of a soluble factor, this appeared to have a positive effect on sprout formation and was able to sustain vessel stability over the course of 7 days (Fig. 3g). Vessel integrity was not as advanced as cultures grown in the presence of SF, and single unattached cells were more frequently seen. Lumen formation was scarce and lumens that did appear were highly irregular in shape. We were concerned that the factor(s) may be labile and so we prepared fresh CM that was then frozen in small aliquots. These were thawed once and added to the cultures every day, however, no difference was observed between fresh CM and normal CM (data not shown). These findings suggest that factor(s) secreted by SF and present in the CM can substitute for the continued presence of SF to an extent, however, CM derived from SF cultured alone does not fully recapitulate the effects of SF that have been grown continuously with EC.

The failure of Ang-1 to support long-term capillary formation in the fibrin gels may be due to the absence of its receptor, Tie-2. We therefore investigated the level of Tie-2 receptor expression in HUVEC using RT-PCR. As shown in Fig. 4, Tie-2 mRNA is present at the initiation of culture and is still present at day 6. In addition, we also observed Tie-2 expression at intermediate stages of sprouting (data not shown). Thus, the failure of Ang-1 to support long-term stability is not explained by lack of the Tie-2 receptor.

We have previously reported that the basic helix-loop-helix (bHLH) transcription factor HESR-1 is rapidly in-

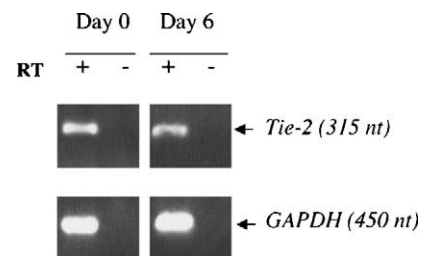


Fig. 4. Tie-2 receptor expression is maintained during sprouting. RT-PCR analysis of *Tie-2* expression at day 0 and day 6. *Tie-2* mRNA is present at both timepoints. (+) and (–) Presence or absence of RT step.

duced in HUVEC during capillary-like network formation in collagen gels and that these cells express the upstream effectors *notch 1*, *notch 4*, and *delta 4* (Taylor et al., 2002). Moreover, it has been shown that these molecules are critically important during development of the cardiovascular system in vivo (Gridley, 1997). To examine expression of these molecules in EC during sprouting in fibrin gels, we purified EC from cultures as described under Materials and Methods, isolated mRNA, and performed RT-PCR. In agreement with our previous data, *HESR-1* expression was upregulated in sprouting EC compared to migrating and proliferating cells growing in 2D cultures (Fig. 5). In addition, we also noted strong induction of *notch 1*, *notch 4*, and *delta 4* in sprouting cells. No product was amplified in the absence of the reverse transcription step (Fig. 5, and data not shown), indicating the absence of genomic contamination. Thus, as we have reported before, the induction of *HESR-1* in these cultures is consistent with activation of the Notch pathway, a conclusion supported by the finding of strong upregulation of both receptor (notch) and ligand (delta).

Finally, a potential strength of in vitro systems compared to in vivo models for angiogenesis is that gene expression in cultured cells is more easily manipulated. In order to efficiently deliver a gene of interest into HUVEC cells, we decided to use a recombinant, replication-deficient adenovirus system. HUVEC growing as a monolayer were infected at different MOIs with adenoviruses carrying a cDNA cassette for GFP. The expression of GFP was monitored 24 h postinfection using flow cytometry. As shown in Fig. 6, the levels of GFP expression, as determined by the mean fluorescence, correlated with the adenovirus MOI, with maximal expression at an MOI of 250. The percentage of cells positive for GFP expression ranged from 95% at an MOI of 7 to 99% at an MOI of 250.

When placed into fibrin gels, cells transfected with Ad-IRES-GFP at high MOIs failed to form sprouts (data not shown). However, at MOIs below 10, sprout formation was

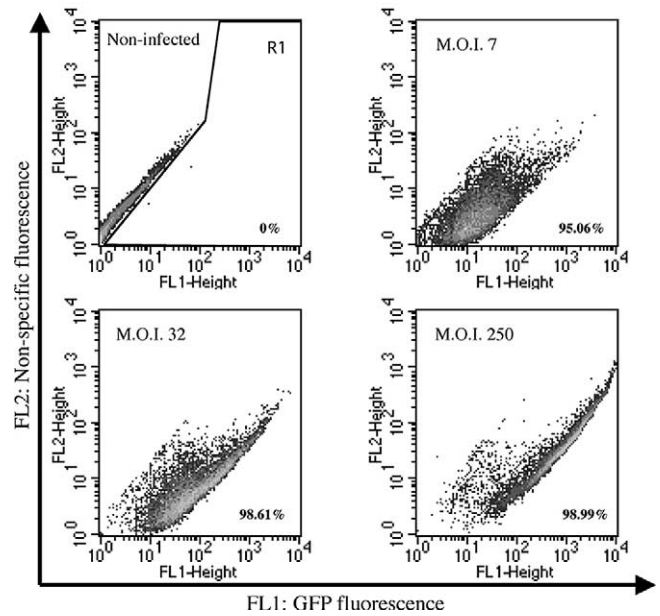


Fig. 6. HUVEC can be infected at high efficiency with a low MOI of adenovirus. HUVEC were infected with the recombinant adenovirus Ad-GFP at the indicated MOI. Twenty-four hours following infection, cells were harvested and GFP expression was analyzed by FACS. GFP expression was evaluated using the FL1 channel (x parameter: FL1-Height-FITC/GFP log), whereas nonspecific fluorescence was evaluated using the FL2 channel (y parameter: FL2-Height-PE log). Values correspond to the percentage of GFP-positive cells. Background fluorescence is present equally in both channels and therefore falls on the diagonal.

vigorous and indistinguishable from that observed with non-infected HUVEC (Fig. 7a). Both the average number of sprouts per bead and the average sprout length remained constant when noninfected and Ad-IRES-GFP infected HUVEC were compared (Fig. 7b). Importantly, as noted above, even at an MOI of 10 or below, greater than 95% of the cells are positive for GFP. Altogether, these experiments demonstrate that the recombinant replication-deficient adenovirus technology can efficiently deliver a gene of interest to HUVEC and is nontoxic at an MOI of 10 or below.

**Discussion**

Although the original fibrin gel protocol of Nehls and Drenckhahn has proven useful for generating capillary-like sprouts from microvascular EC, that protocol does not support the growth of HUVEC (von Bulow et al., 2001). Given the ready availability, ease of preparation and extensive literature on these cells, we chose to develop a modified procedure that allows rapid sprouting of HUVEC in fibrin gels and development of complex capillary networks. In sharp contrast to many previously published methods, our modified procedure reliably produces long vascular sprouts with clearly defined lumens surrounded by multiple, polarized EC that are similar to those observed in vivo. In

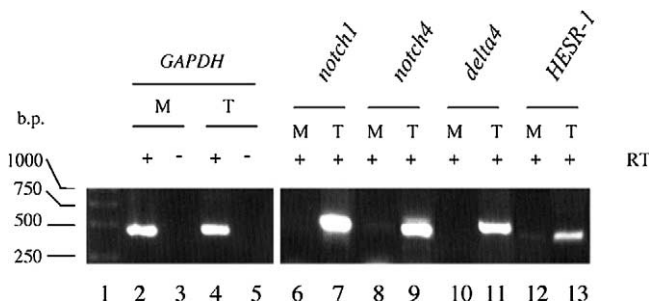


Fig. 5. Notch pathway genes are expressed in sprouting EC. RT-PCR analysis of *notch1* (lanes 6 and 7), *notch4* (lanes 8 and 9), *delta4* (lanes 10 and 11), and *HESR-1* (lanes 12 and 13) expression in HUVEC monolayer (M) versus tube-forming HUVEC in fibrin gels (T). *GAPDH* levels were similar in monolayer and in tubes (lanes 2 and 4). No *GAPDH* band was detected in the absence of RT (lanes 3 and 5). Molecular weight marker is presented in lane 1.

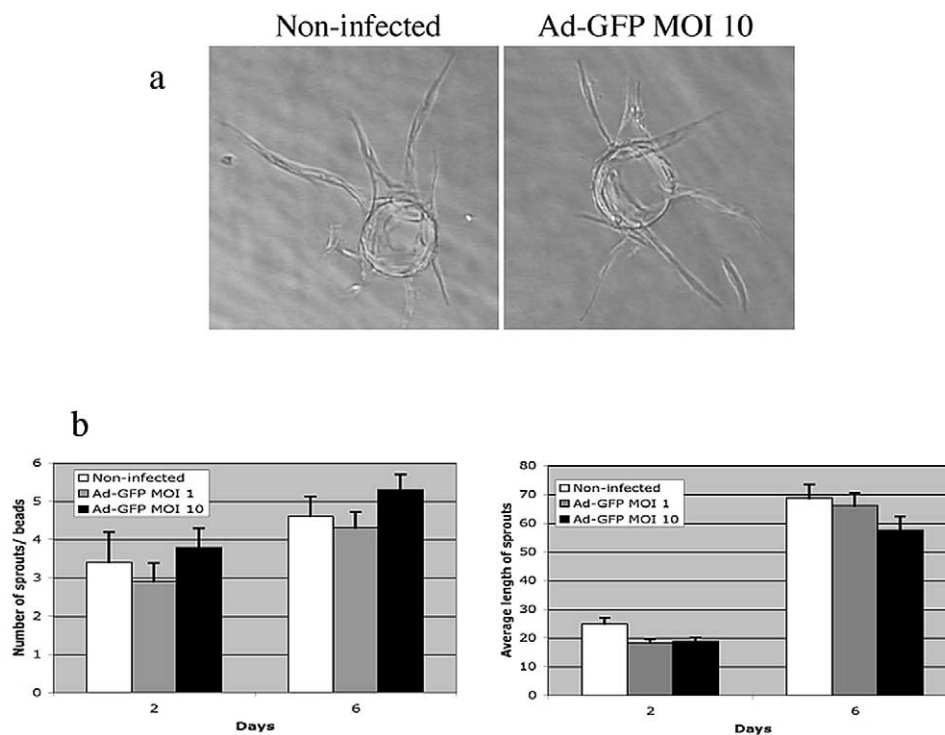


Fig. 7. Adenovirus infection at low MOI does not affect sprouting EC. (a) Both noninfected HUVEC (left panel) and Ad-IRES-GFP-infected HUVEC (MOI of 10, right panel) produce robust sprouts. At this MOI more than 95% of the cells are GFP positive. (b) The average number of sprouts per bead (left panel) and the average length of sprouts (right panel) in bead assays using noninfected HUVEC, or HUVEC infected at an MOI of 1 and 10. Average sprout length is in arbitrary units.

addition, these sprouts branch and anastomose to form capillary networks.

Several groups have demonstrated the ability of mesenchymal cells such as fibroblasts to stabilize vascular sprouts *in vitro* (Montesano et al., 1993; Tille and Pepper, 2002; Velazquez et al., 2002; Villaschi and Nicosia, 1994), although the factor(s) responsible for this has not been definitively identified. In the cocultures, SF are grown on top of the gels, which are 3–4 mm thick, and we have never seen migration of the cells down to the level of the beads (which sink to the bottom of the well before the fibrin sets). Thus we do not believe that EC-SF contact is required for induction of stability, though this has not been conclusively ruled out. Our data do suggest, however, that there may be cross-talk between EC and SF in these cultures (see below). Interestingly, a previous report using bovine EC-coated beads in fibrin gels found no evidence for stabilization of networks by fibroblasts (Nehls and Drenckhahn, 1995) and we have confirmed the result. This may be due to differences in the fibroblasts used (bovine corneal versus human skin) or may reflect more stringent growth factor requirements for HUVEC. Similar findings of fibroblast-dependent vessel growth were reported by Velazquez et al. in collagen gels. Here characteristic disconnected capillaries were observed in the absence of fibroblasts. Upon the addition of fibroblasts, however, EC formed tubular structures that invaded the collagen gel. Surprisingly, EC-fibroblast contact

was required for capillary formation. We also find that membranes with 0.4  $\mu\text{M}$  pore size placed between the EC and SF block sprouting (data not shown), however, as we see absolutely no evidence for EC-SF contact, we interpret this result to show that the membrane provides too great a barrier for diffusion of essential factors.

Additional data on the role of mural cells, such as SMC, in providing stabilization signals to sprouting EC have been provided (Benjamin et al., 1998; Darland and D'Amore, 2001; Orledge and D'Amore, 1987). Recent evidence suggests cell-cell interactions after mural cell recruitment are important in vascular remodeling. For example, in developing retinas EC are associated with  $\alpha$ -smooth muscle actin-positive pericytes. This association could be blocked by intraocular injection of platelet-derived growth factor-(PDGF) BB, leading to a loss of vessel stabilization and major vessel regression (Benjamin et al., 1998). Similar results were observed by blocking EC-mural cell interactions through injection of an antagonistic mAb against PDGFR- $\beta$  (Uemura et al., 2002). Addition of recombinant Ang-1 rescued the abnormal vessel phenotype even in the absence of pericytes. Finally, addition of TGF- $\beta$  to cocultures of EC and 10T1/2 cells in Matrigel induces capillary-like structures. Upon blocking of the TGF- $\beta$  signal by specific inhibitors, vessel formation is also blocked (Darland and D'Amore, 2001).

We tested two factors previously identified as proangio-

genic (Ang-1), which promotes sprouting and vessel stabilization (Koblizek et al., 1998; Papapetropoulos et al., 1999), and TGF- $\beta$ , which can be either pro- or antiangiogenic depending on context (Madri et al., 1988; Pepper, 1997). Although TGF- $\beta$  promotes angiogenesis in vivo, there are conflicting data in vitro, and indeed, our results concur. We found that TGF- $\beta$  was not able to replace SF in our assay, neither did it promote sprout formation beyond what was seen in control cultures. We therefore conclude that TGF- $\beta$  is unlikely to be a major fibroblast-derived contributor to sprout formation in vitro. Our results with Ang-1 were surprising. It has been shown directly in vitro, and inferred in vivo, that Ang-1 induces EC sprouting (Koblizek et al., 1998; Suri et al., 1996), but it also has a critical role in recruitment of pericytes to developing vessels, which then act to stabilize the vessels and induce a nonleaky phenotype (Suri et al., 1996). In vitro, Ang-1 has also been shown to stabilize primitive vascular networks in collagen gels in the absence of stromal cells, suggesting a direct effect; however, the increased stability was only effective up to 48 h (Papapetropoulos et al., 1999). Our results are in line with this in that Ang-1 induced enhanced sprouting at early times but was not able to promote vessel stabilization beyond 2–3 days. These data suggest that Ang-1 does not stabilize developing blood vessels directly but rather it acts indirectly by promoting recruitment of support cells, which then provide additional signals. In light of the recent data showing Ang-1 stabilization of vessels in the absence of stromal cells in vivo (Uemura et al., 2002), it may also be possible that Ang-1 fails to stabilize vessels in the continued presence of VEGF. Potentially, in the absence of continuous proangiogenic signaling by VEGF, Ang-1 may promote vessel stability. Indeed, in cultures where VEGF was not replaced after 7 days, vessels did not regress but rather maintained a mature phenotype with a well-developed lumen. Further studies will be needed to determine whether the Ang-1 being made by the SF is sufficient to stabilize the vessels in the absence of VEGF.

Other growth factors that influence vessel function include the platelet-derived phospholipids phosphatidic acid and sphingosine 1-phosphate (Sph-1-P). Both factors, upon stimulation of platelets, are released and are suggested to be involved in vessel maturation (English et al., 1999). Sph-1-P has been shown to function in numerous EC processes during angiogenesis and also serves as a chemoattractant that induces EC proliferation and differentiation (English et al., 2000). A recent report demonstrates that Sph-1-P enhances vessel integrity by directly inhibiting EC proliferation and switching the EC phenotype to a less permeable barrier, thereby decreasing vessel leakage (Garcia et al., 2001). This is strikingly similar to the effects of Ang-1 and both factors may act together in stabilizing vessel integrity. Further experiments will be necessary to examine the role of Sph-1-P in capillary maturation in fibrin gels.

SF CM appears to stimulate vessel growth and maintain vessel integrity, but to a lesser degree than SF that contin-

uously share medium with the EC. This may be due to the factor(s) being labile—not surviving a freeze–thaw cycle—or they may not be released into the medium. Many growth factors have a strong affinity for extracellular matrix proteins, including some isoforms of VEGF, and may not, therefore, accumulate in the medium. Related to this, it is also possible that the factor is secreted only from the basal side of the SF, directly into the fibrin gel. Finally, release of the appropriate factors may be dependent on the SF receiving inducing signals from the EC. Such cross talk between EC and stromal cells has been proposed previously (Davis and Yancopoulos, 1999).

Our previous work has demonstrated an important role for the bHLH transcription factor HESR-1 in EC network formation in collagen gels (Taylor et al., 2002). Moreover, we have shown that the Notch pathway is an upstream effector of HESR-1 expression and that overexpression of this transcription factor downregulates the VEGFR-2 promoter and VEGFR-2-mediated EC proliferation. In the more sophisticated in vitro assay we have described here, we also see upregulation of *HESR-1* expression, indicative of Notch signaling. Interestingly, we also see a strong upregulation of notch and notch ligand expression in the fibrin gels, a result we did not see in collagen gels. Increased expression of *notch 1*, *notch 4*, and *jagged* has recently been demonstrated at the regenerating endothelial wound edge in damaged vessels (Lindner et al., 2001), suggesting that the use of fibrin gels may represent a more physiological model than the collagen gel system.

We believe that this assay compares favorably with several other in vitro angiogenesis assays. In previous work, from our lab and others, capillary-like vessels growing in collagen gels tended to be thin and have intracellular lumens that appear to form from vacuoles. In many cases the lumen was completely enclosed in a single EC—a so-called seamless capillary. Similar, immature capillaries form as “cords” on Matrigel. Both assays appear to involve coalescence of EC into cordlike structures and do not model sprouting as such. In addition, network formation on Matrigel is extremely rapid and does not require either transcription or protein synthesis and is independent of cell proliferation (Laterra and Goldstein, 1991; Zimrin et al., 1995). The use of the tumor promoter and PKC agonist PMA in conjunction with fibrin gels promotes more mature capillaries (Nehls and Drenckhahn, 1995), but in our hands induces only short fragments of vessels. In addition, the use of a strong agonist such as PMA makes the system less than ideal for studying the role of different signaling pathways. One of the best in vitro assays uses aortic rings embedded in collagen or fibrin. Although this results in vigorous sprouting and is easily quantifiable it is difficult to genetically modify the cells, and the presence of multiple, closely apposed, cell types makes it difficult to determine whether manipulations that affect the sprouting phenotype are the result of targeting EC, support cells, or both. In contrast, the fibrin gel assay described here has multiple advantages: it

uses HUVEC, which are easily obtainable and well-characterized; it generates mature, complex vessels; it is easy to quantitate; and, it allows easy manipulation of gene expression using either transfection or adenoviral delivery. Although it is true that *in vivo* assays provide the most rigorous test of hypotheses, we believe that this assay provides an excellent approximation to *in vivo* angiogenesis as well as significant advantages.

In summary, we describe a modified *in vitro* angiogenesis assay that recapitulates several of the steps known to be crucial to new blood vessel formation *in vivo*.

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