Endothelial cell spheroids as a versatile tool to study angiogenesis *in vitro*

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ABSTRACT **Given the need for robust and cost-efficient *in vitro* models to study angiogenesis and reproducibly analyze potential pro- and antiangiogenic compounds in preclinical studies, we developed a 3–dimensional *in vitro* angiogenesis assay that is based on collagen gel-embedded, size-defined spheroids generated from cultured human umbilical vein endothelial cells (HUVECs). Despite its wide distribution, limitations, sensitivity, robustness, and improvements, the capacity of this assay for functional screening purposes has not been elucidated thus far. By using time-lapse video microscopy, we show that tip cells lead the formation of capillary-like and partially lumenized sprouts originating from the spheroids. Angiogenic sprouting from spheroids generated from 5 different primary cultured human endothelial cell types was induced by physiologic concentrations of vascular endothelial cell growth factor 165. Based on this assay system, we determined the capacity of 880 approved drugs to interfere with or boost angiogenic sprouting, thereby assessing their putative angiogenesis-related side effects or novel applications. However, although this assay allowed for a rapid and reproducible determination of functional IC_{50} values of individual compounds, the sprouting results were partially affected by the HUVEC passage number and donor variability. To overcome this limitation, immortalized HUVECs (iHUVECs) showing a more homogenous response in terms of proliferation and sprouting over multiple population doublings were used in the course of this study. Collectively, the spheroid-based angiogenesis assay provides a sensitive and versatile tool to study the impact of pro- and antiangiogenic determinants on multiple steps of the angiogenic cascade. It is compatible with different endothelial cell types and allows use of iHUVECs to improve its overall robustness.—Heiss, M., Hellström, M., Kalén, M., May, T., Weber, H., Hecker, M., Augustin, H. G., Korff, T. Endothelial cell spheroids as a versatile tool to study angiogenesis *in vitro*. *FASEB J.* 29, 3076–3084 (2015). www.fasebj.org**

Key Words: HUVEC · VEGF · tip cells

GROWTH AND EXTENSION OF THE capillary network is rate limiting for a plethora of physiologic and pathophysiologic processes such as tissue or tumor growth, wound healing, compensation of ischemia, and inflammation. Sprouting and elongation of new capillaries from preexisting networks are critical steps within a cascade of events referred to as angiogenesis. In a nutshell, proangiogenic growth factors such as vascular endothelial cell growth factor (VEGF) stimulate quiescent endothelial cells to form tip cells, which form filopodia (1), proteolytically degrade their surrounding extracellular matrix, and migrate toward the angiogenic stimulus. Following (stalking) cells proliferate to form new capillary-like sprouts, which eventually become lumenized and covered by pericytes. As a consequence, endothelial cells usually maintain their quiescent phenotype, which is controlled by the dynamic balance between stimulators and inhibitors of angiogenesis (2).

To mimic angiogenesis *in vitro*, a plethora of artificial assay systems has been developed focusing on individual aspects of the angiogenic cascade (3, 4). They are all based on the culture of endothelial cells on plastic surfaces to generate a monolayer of cells that mimics many of the functional properties of the endothelium *in vivo* (5). However, when maintained in standard 2-dimensional cell culture, endothelial cells tend to progressively lose their differentiated phenotype as can exemplarily be deduced from the loss of CD34 expression under such conditions (6, 7). Furthermore, as cell culture conditions are selected so that endothelial cells can grow, they usually do not support the quiescent, resting phenotype that these cells maintain

Abbreviations: Ang-2, angiopoietin-2; CSL, cumulative sprout length; ECGM, endothelial cell growth medium; FGF-2, fibroblast growth factor-2; HUVEC, human umbilical vein endothelial cell; iHUVEC, immortalized human umbilical vein endothelial cell; PDGFRβ, platelet-derived growth factor receptor β; VEGF, vascular endothelial cell growth factor

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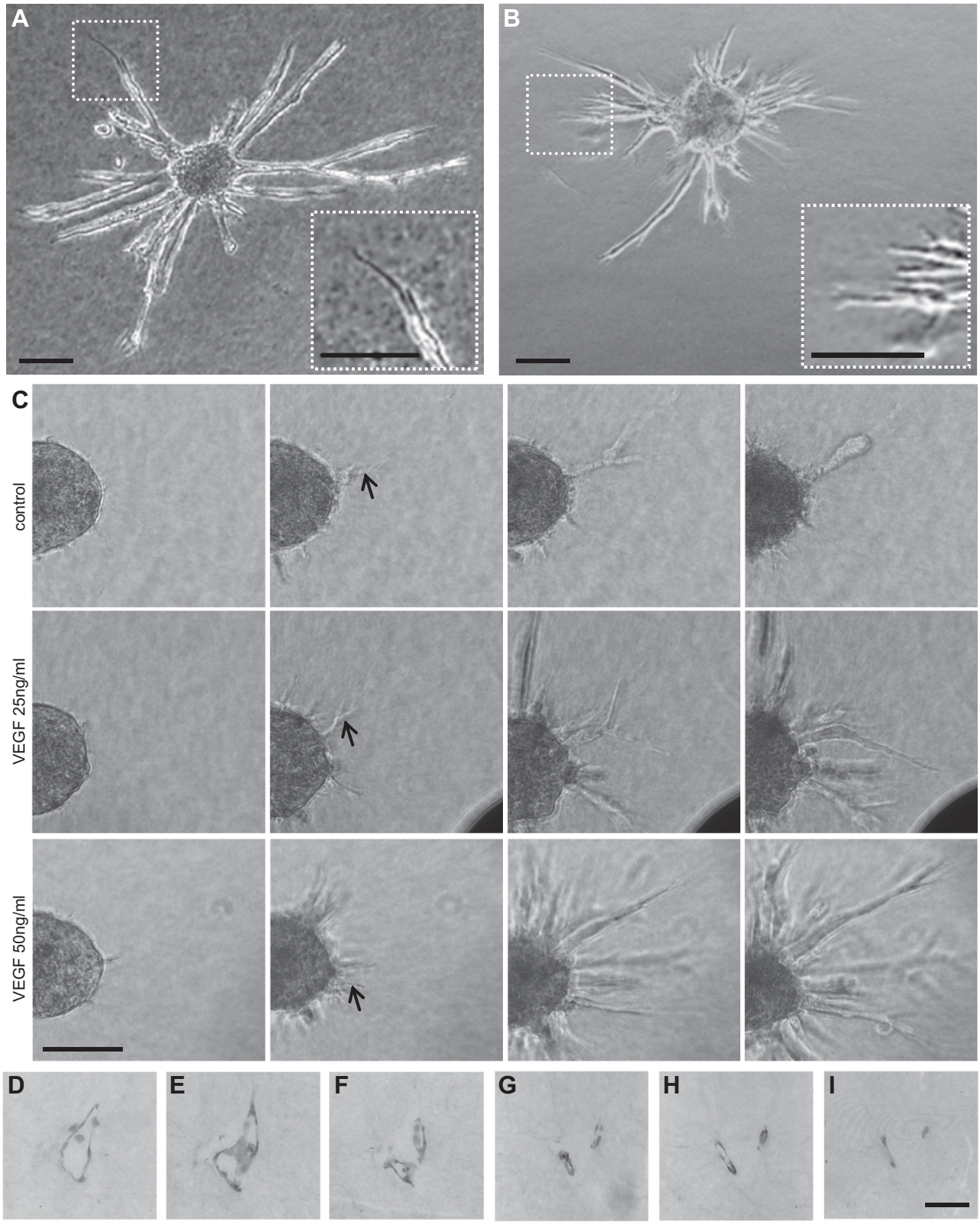

Figure 1. Analysis of endothelial cell sprouts originating from collagen-embedded HUVEC spheroids. HUVEC spheroids were embedded in collagen gels and stimulated with 50 ng/ml FGF-2 (*A*) or VEGF (*B*). After 36 hours, branching capillary-like sprouts originated from the spheroid body and invaded the collagen matrix. *A, B*) The leading cells extended filopodia into the collagen-gel. Scale bars, 100 μm. *C*) Time-lapse video microscopy images (20 hours) indicated filopodia formation of the tip cells (arrows). Scale bar, 100 μm. Paraffin serial sections of sprouts, originating from collagen embedded spheroids, revealed that capillary-like sprouts form continuous lumen (*C–H*; scale bar, 20 μm) dividing at the branching points (*E, F*).

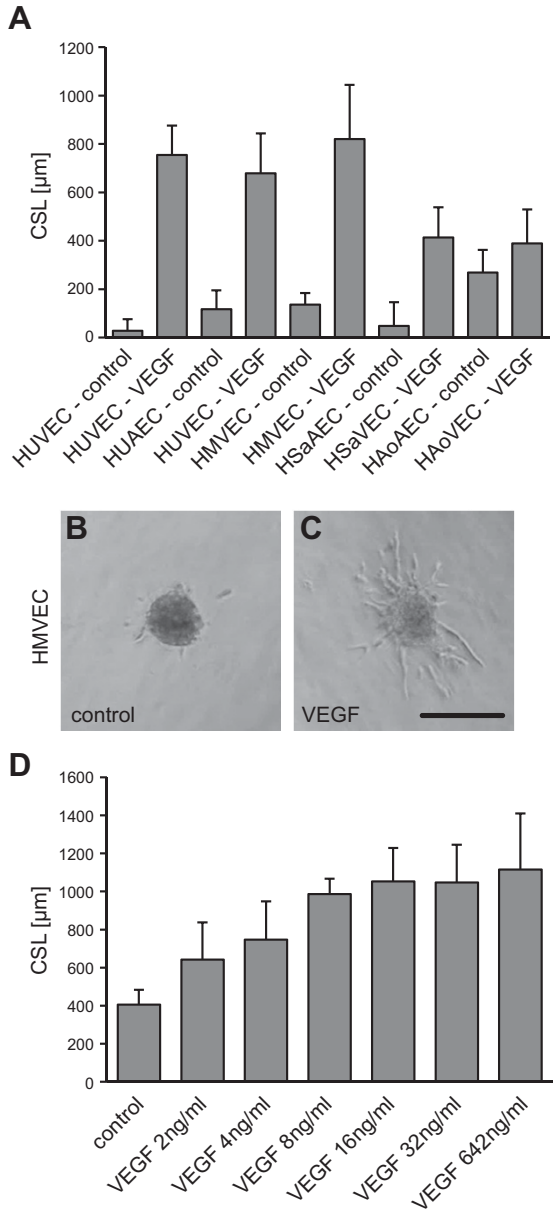

Figure 2. Analysis of capillary sprouting of endothelial cells from different origin. *A*) Collagen gel-embedded spheroids of different endothelial cell types were stimulated with 25 ng/ml VEGF for 24 hours. $^{*}P < 0.05$ *vs.* corresponding controls. *B, C*) Representative spheroids of human dermal microvascular endothelial cells are shown. Scale bar, 200 µm. Collagen-embedded spheroids of HUVECs were stimulated with increasing doses of VEGF for 24 hours (*D*; $^{*}P < 0.05$ *vs.* control). Mean CSL was calculated for 10 randomly selected spheroids per experimental group.

in vivo (8). This limitation has been partially overcome by 3-dimensional culture techniques allowing the endothelial cells to acquire a resting phenotype within size- and cell number-defined spheroids (9). Based on those defined endothelial cell aggregates, a widely used *in vitro* angiogenesis assay was developed that represents all steps of capillary sprout formation (10, 11). For instance, it was applied to delineate the impact of angiopoietin-2 (Ang-2) on angiogenic sprouting (12), to identify novel anti- and proangiogenic determinants (13, 14), to characterize the relevance of miRNAs for angiogenesis (15), or to reveal molecules controlling capillary sprout formation on the epigenetic level (16). This assay technique was further exploited to engineer a human vasculature in mice (17), which allows emulating human vascular diseases such as cerebral cavernous malformation (18). Recently, this assay has been used to verify SYNJ2BP as a novel inhibitor of tip cell formation by promoting Delta-Notch signaling (19).

Despite the versatility of this spheroid-based angiogenesis assay, its sensitivity and usability for screening purposes was never explored in detail. To this end, this study was aimed at investigating the advantages and limitations of the spheroid-based angiogenesis assay and its capacity to determine functional IC_{50} values of antiangiogenic drugs. Furthermore, the capacity of iHUVECs to serve as a stabilized source of endothelial cells was evaluated.

MATERIAL AND METHODS

Antibodies, growth factors, and reagents

Human recombinant fibroblast growth factor-2 (FGF-2) and VEGF were obtained from R&D Systems (Wiesbaden, Germany). Methylcellulose (4000 centipoises, catalog no. M-0512) was from Sigma-Aldrich (Deisenhofen, Germany). Primary antibodies for immunohistochemistry were from Santa Cruz Biotechnology

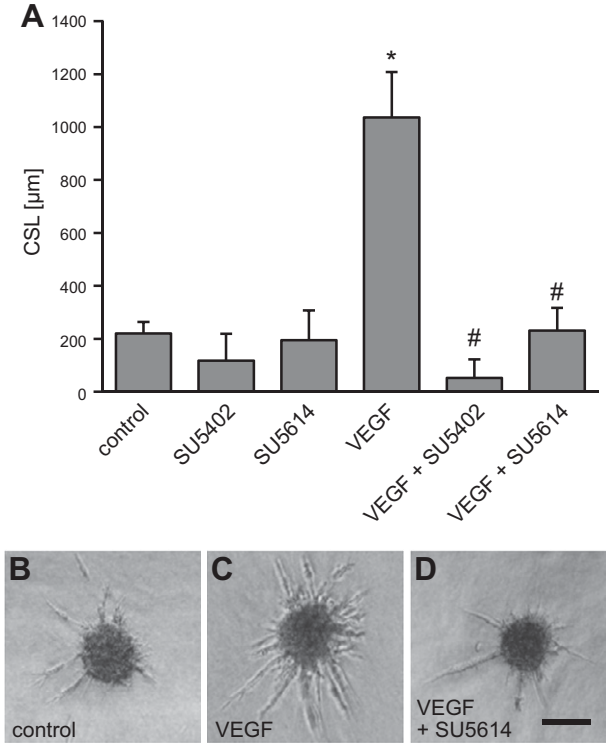

Figure 3. Analyzing the effects of the antianiogenic compounds SU5402 and SU5614. *A*) The kinase inhibitors SU5402 (10 µM) and SU5614 (10 µM) were analyzed for their capacity to inhibit VEGF-induced (25 ng/ml, 24 hours) HUVEC sprouting. $^{*}P < 0.05$ *vs.* control, $^{\#}P < 0.05$ *vs.* VEGF. *B–D*) Representative spheroids are shown. Scale bar, 100 µm). Mean CSL was calculated for 10 randomly selected spheroids per experimental group.

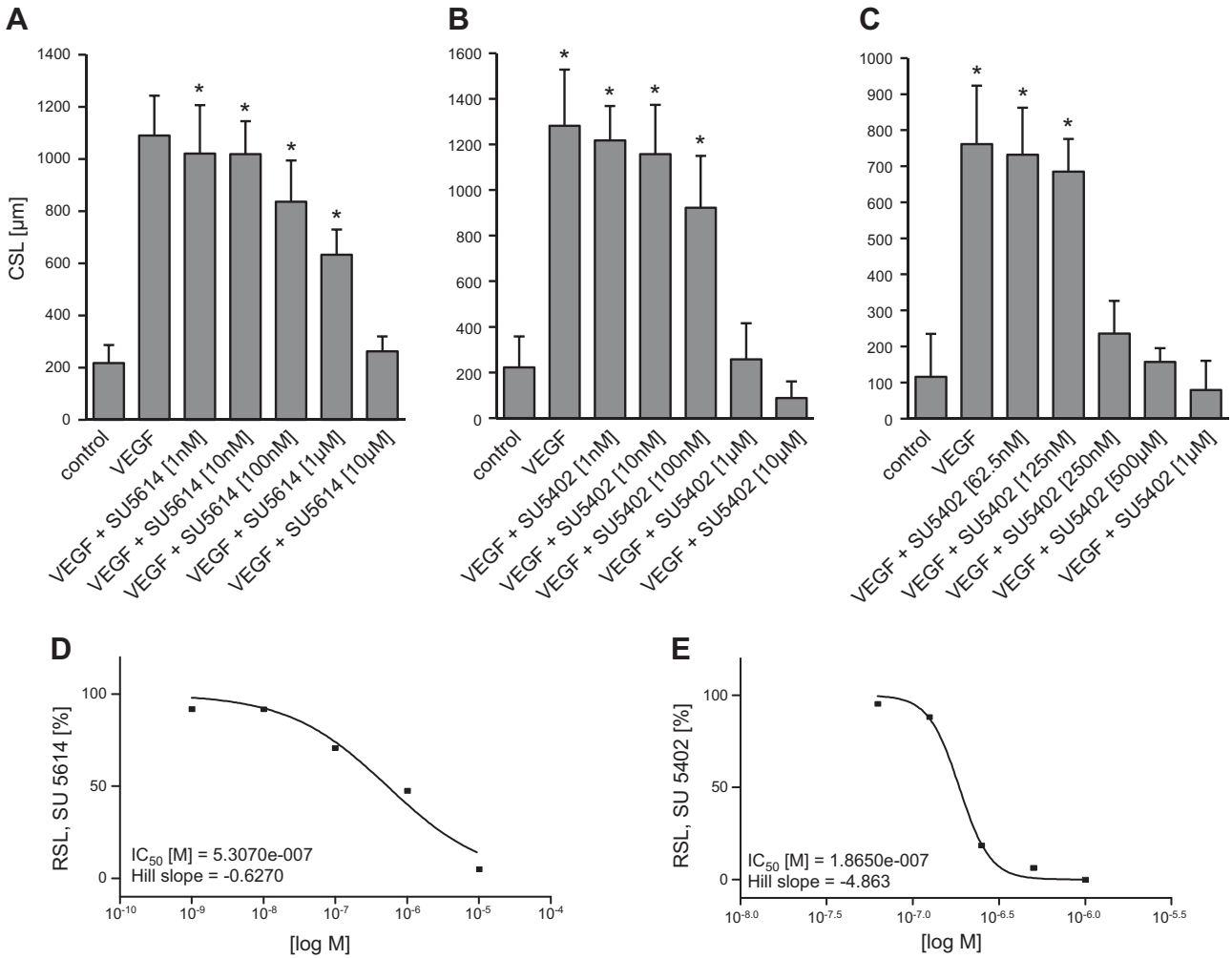

Figure 4. Determination of the functional IC_{50} values of SU5402 and SU5614. *A, B*) The capacity of kinase inhibitors to inhibit VEGF-induced capillary sprouting was titrated. $^*P < 0.05$ *vs.* control. *C*) SU5402 titration was fine-tuned by a second titration analysis. $^*P < 0.05$ *vs.* control. *D, E*) IC_{50} values were calculated based on the relative sprout length, with VEGF-induced CSL set to 100% and baseline sprouting set to 0%. Mean CSL was calculated for 10 randomly selected spheroids per experimental group.

(anti-VE-Cadherin antibody sc6458; Dallas, TX, USA) and from R&D Systems (anti-Ang-2 antibody AF623) and used according to the manufacturer's recommendations. The donkey anti-goat-Cy5 antibody was from Dianova (Hamburg, Germany). DAPI was obtained from Life Technologies (Carlsbad, CA, USA). Mowiol was purchased from Calbiochem (Hilden, Germany).

Cell culture

Endothelial cell growth medium (ECGM) and endothelial cell growth supplement (human umbilical vein endothelial cell culture) were purchased from Promocell (Heidelberg, Germany). Fetal bovine serum was obtained from Biochrom (Berlin, Germany). Human umbilical vein endothelial cells (HUVECs) were freshly isolated from individual umbilical cord veins of newborn babies by collagenase digestion. Cells were cultured at 37°C in ECGM containing 10% heat-inactivated fetal bovine serum and frozen in liquid nitrogen at passage 2 or 3. Only HUVECs cultured up to passage 6 were used for experiments. Human umbilical artery, dermal microvascular, aortic, and saphenous vein endothelial cells were purchased from Promocell and cultured at 37°C according to the manufacturer's instructions. Immortalized HUVECs (iHUVECs, INS-CI-1001; HKO) were kindly provided by InSCREENeX GmbH (Braunschweig, Germany) and cultured under the same conditions as the HUVECs. Human fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum.

Generation of endothelial cell spheroids

Endothelial cell (or fibroblast) spheroids of defined cell number were generated as described previously (9). In brief, HUVECs or other endothelial cell types were suspended in corresponding culture medium containing 0.25% (w/v) methylcellulose and seeded in nonadherent round bottom 96-well plates (Greiner, Frickenhausen, Germany). Under these conditions, all suspended cells contribute to the formation of a single spheroid per well of defined size and cell number (*in vitro* angiogenesis: 500 cells per spheroid). Spheroids were cultured for ≥24 hours and used for the corresponding experiments.

In vitro angiogenesis assay

For the *in vitro* angiogenesis assay, spheroids were generated overnight, after which they were embedded into collagen gels. A collagen stock solution was prepared prior to use by mixing

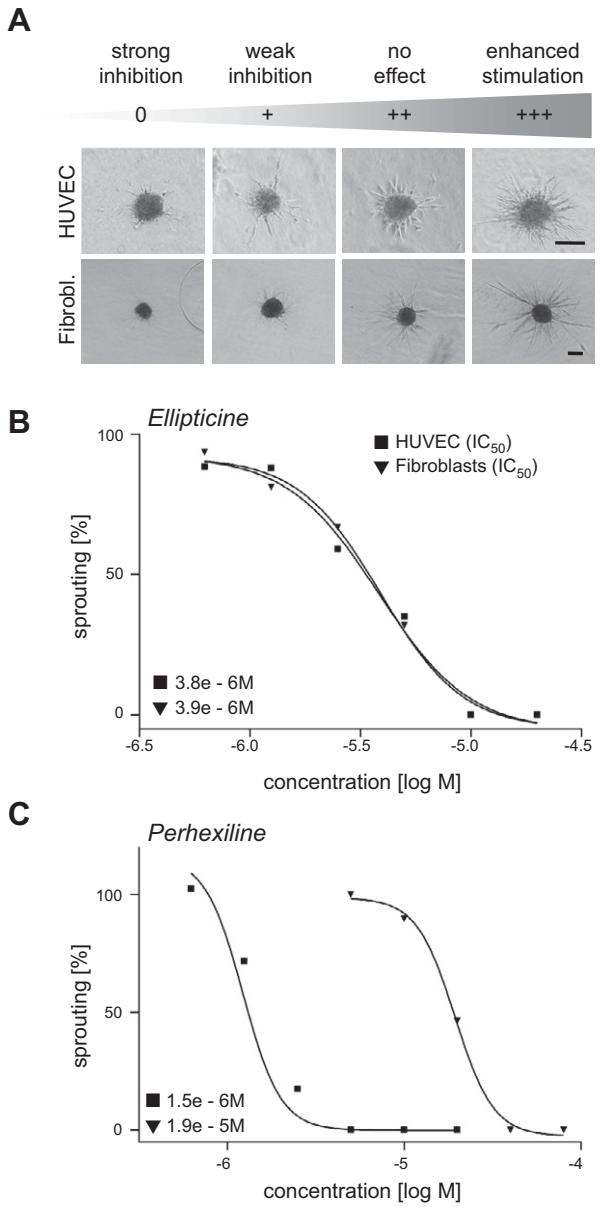

Figure 5. Screening of 880 drugs for their anti- or proangiogenic capacity. HUVEC sprouting was induced by VEGF (25 ng/ml; baseline sprouting: ++). *A*) Drug-dependent alterations in the sprouting intensity were ranked from zero (0, no or strongly reduced sprouting activity compared with baseline) to triple cross (+++, enhanced sprouting activity compared with baseline). Based on this ranking, 880 drugs (10 μM) were screened for their capacity to inhibit or to enhance VEGF-stimulated HUVEC sprouting. In a second approach, all compounds that had shown an effect were screened for their capacity to inhibit or stimulate fibroblast baseline sprouting (*A*; scale bars, 100 μm). Forty compounds completely inhibited VEGF-induced HUVEC sprouting, from which 12 did not or only minimally affected fibroblast sprouting. Forty-three compounds weakly inhibited HUVEC sprouting, from which 34 did not affect fibroblast sprouting. Nineteen compounds enhanced VEGF-stimulated HUVEC sprouting from, which 11 did not stimulate fibroblast sprouting. *B*) Compounds such as ellipticine, which inhibited both HUVEC and fibroblast sprouting, were considered nonspecific (see Table 1). *C*) Compounds such as perhexiline, which preferentially affected HUVEC sprouting, were considered specific.

8 volumes of acidic collagen extract of rat tails (equilibrated to 2 mg/ml, 4°C) with 1 volume of 10 × M199 (Gibco BRL, Eggenstein, Germany), and ~1 volume of 0.2 N NaOH to adjust the pH to 7.4. This stock solution (4 ml) was mixed with 4 ml ECGM basal medium (without supplements) containing 20% fetal calf serum (Biochrom, Berlin, Germany) and 0.5% (w/v) methylcellulose to prevent sedimentation of spheroids prior to polymerization of the collagen gel. The spheroid-containing gel (~50 spheroids/ml) was rapidly transferred into prewarmed 24-well plates and allowed to polymerize (30 minutes), after which 0.1 ml ECGM basal medium was pipetted on top of each gel containing the test substances. The gels were incubated at 37°C, 5% CO_2, and 100% humidity. After 24 hours, *in vitro* angiogenesis was quantitated digitally by measuring the length and number of the sprouts (calculated as cumulative sprout length) that had grown out of each spheroid (×10 objective magnification) using the digital imaging software Cell (Olympus, Hamburg, Germany) analyzing ≥10 spheroids per experimental group and experiment. In the context of the screening approach, sprouting was evaluated by roughly grading the sprouting responses (0, no sprouting; +, weak sprouting; ++, robust sprouting; +++, intense sprouting).

Immunofluorescence analyses

Cells were fixed in ice-cold methanol for 15 minutes and allowed to dry for 20 minutes. Rehydrated cells were blocked with 0.25% casein and 0.1% bovine serum albumin for 30 minutes. Cells were incubated with anti-VE-cadherin or anti-Ang-2 antibody at 4°C overnight. After washing, cells were incubated with Cy5 labeled secondary antibody for 1 hour and mounted with Mowiol. Nuclei were visualized by counterstaining the cells with DAPI. Fluorescence was recorded using an Olympus IX3 fluorescence microscope (Olympus).

Statistical analysis

If not otherwise indicated, all results are expressed as means ± SD of 1 of 2 or 3 experiments with comparable results determining the cumulative sprout length (CSL) of 10 randomly selected spheroids per experimental group. Differences between 2 matched experimental groups were statistically analyzed by unpaired Student *t* test (InStat 3.0 or Prism 6; GraphPad Software, La Jolla, CA, USA) with a probability value of $P < 0.05$ considered statistically significant. Differences among 3 or more experimental groups were analyzed by 1-way ANOVA followed by a Tukey *post hoc* test (InStat 3.0 or Prism 6; GraphPad Software), with a probability value of $P < 0.05$ considered statistically significant.

RESULTS

Single tip cells lead capillary sprouts originating from growth factor-stimulated spheroids

HUVEC spheroids are widely used to analyze angiogenic activation of endothelial cells *in vitro*. Recent publications

TABLE 1. *Summary of screening results*

Effect	Strong inhibition	Weak inhibition	Stimulation
Total	40	43	19
Specific	12	34	11

also indicate that this assay may provide a tool to study tip cell formation (19). To prove whether spheroid-based sprout formation is initiated and guided by a defined tip cell, the morphology of spheroid capillary sprouts was analyzed in detail. We realized that endothelial cells leading FGF-2- or VEGF-induced capillary sprouts extend filopodia into the collagen gel (**Fig. 1*A, B***). Moreover, time-lapse video microscopy analyses suggest that sprouting is initiated by a single cell protruding filopodia into the collagen gel (Fig. 1*C*, arrows) to even the path for following cells (Fig. 1*C* and Supplemental Movie 1). Serial cross sections through collagen gels revealed that capillary sprouting sometimes supports the formation of extended lumenized structures (Fig. 1*D–I*).

Angiogenic sprouting is induced in spheroids from different primary endothelial cell types by low doses of growth factors

To prove whether this assay is compatible with human endothelial cell types other than HUVEC, endothelial cell spheroids were generated from human umbilical artery endothelial cells, human dermal microvascular endothelial cells, human saphenous vein endothelial cells, and human aortic endothelial cells. On stimulation with VEGF, comparable capillary-like sprouts were originating from all the endothelial cell spheroids tested within 24 hours (**Fig. 2*A***). Further experiments were aimed at investigating the sensitivity of this assay for the prototypic proangiogenic growth factor VEGF. Titration experiments showed that VEGF may significantly elicit angiogenic sprouting with doses down to 4 ng/ml or 0.1 nM (Fig. 2*D*) and sometimes even 1 ng/ml depending on the baseline sprouting activity (data not shown).

Identification of antiangiogenic properties of drugs by the spheroid-based angiogenesis assay

Angiogenic sprouting often serves as an adequate *in vitro* parameter to investigate the anti- or proangiogenic capacity of distinct molecular effectors. Here, we assessed the antiangiogenic capacity of 2 small kinase inhibitors, SU5402 (20), an inhibitor of FGF receptor 1, VEGF receptor 2 (VEGFR2), and platelet-derived growth factor receptor β (PDGFRβ) signaling, and SU5614 (21), an inhibitor of VEGFR2 and PDGFRβ signaling. With respect to their inhibitory capacity for the receptor tyrosine kinase VEGFR2, IC_{50} values of about 20 nM and 1.2 μM have been reported for SU5402 and SU5614, respectively. On initial verification of the functional inhibitory capacity of these compounds (**Fig. 3*A–D***), functional IC_{50} values were calculated by titration experiments that stepwise minimized the dosage range (**Fig. 4*A–E***). Despite using several HUVEC preparations during this series of experiments, which showed different baseline sprouting activity (compare Fig. 4*B, C*), relative inhibitory effects of individual compounds were reliably quantified (Fig. 4*C*), underlining the reproducibility of data generated by applying this assay. Based on these results, antiangiogenic IC_{50} values of 0.2 and 0.5 μM were determined for SU5402 and SU5614, respectively. To underline the versatility of this

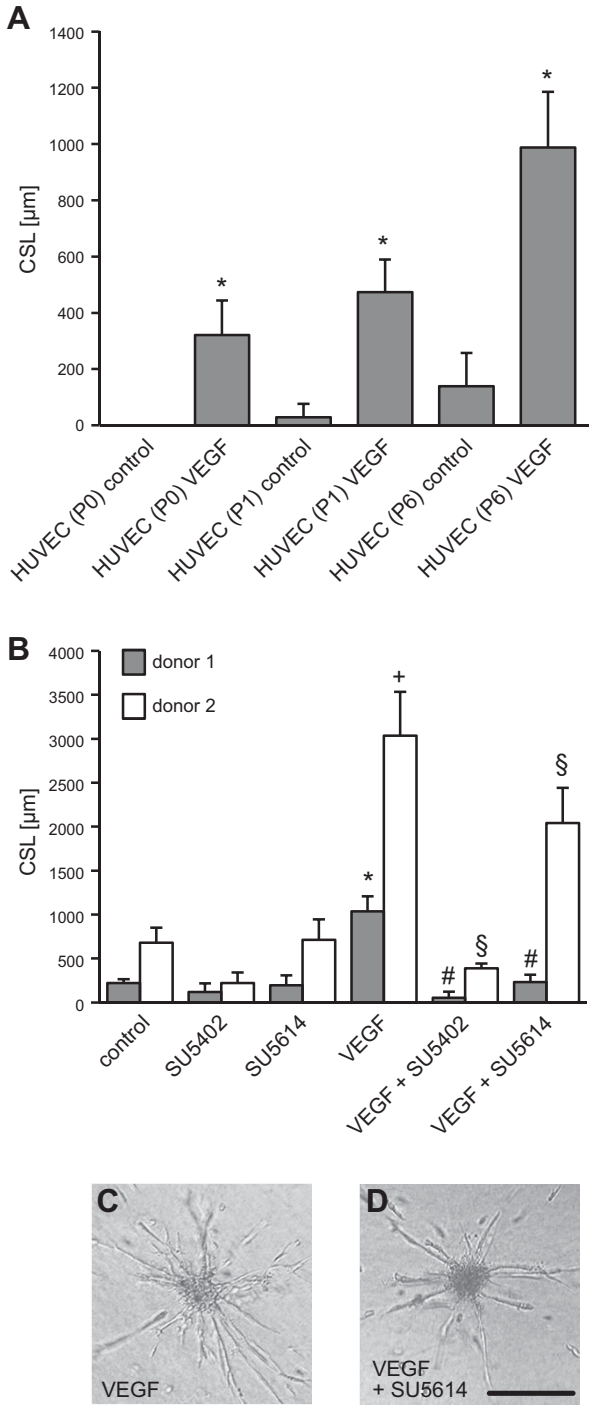

Figure 6. Impact of culture time and donor variability on HUVEC sprouting. *A*) Sprouting from spheroids generated from P0 (freshly prepared), P1, and P6 HUVECs was stimulated with VEGF (25 ng/ml) for 24 hours. $^{*}P < 0.05$ *vs.* corresponding controls. *B*) HUVECs from 2 different donors were stimulated with VEGF (25 ng/ml), SU5402 (10 μM), and SU5614 (10 μM) for 24 hours. $^{*}P < 0.05$ *vs.* control (donor 1), $^{\#}P < 0.05$ *vs.* VEGF (donor 1), $^{+}P < 0.05$ *vs.* control (donor 2), $^{\S}P < 0.05$ *vs.* VEGF (donor 2). *C, D*) Representative spheroids of donor 2 are shown. Scale bar, 200 μm. Mean CSL was calculated for 10 randomly selected spheroids per experimental group.

experimental setup, we screened 880 drugs for their capacity to inhibit or support VEGF-induced angiogenic sprouting. General and therefore nonspecific effects of these drugs on endothelial sprout formation were identified by analyzing their impact on the outgrowth of fibroblasts from corresponding spheroids (**Fig. 5*A*** and **Table 1**). Although most drugs had no effect on the CSL of endothelial cells or inhibited both endothelial and fibroblast sprouting (Fig. 5*B*), some of them such as the antianginal drug Perhexiline appeared to preferentially inhibit endothelial cell sprouting (Fig. 5*C*; for details, see Supplemental Fig. 1).

HUVECs constitute a cellular source for the spheroid-based angiogenesis assay with variable responsiveness

Despite its considerable reliability, the outcome of this assay is limited by the responsiveness of the cultured endothelial cells. To evaluate the variability of this assay with respect to the number of standard cell culture passages (split ratio 1:3), spheroids generated from P6, P1, and P0 (freshly prepared) HUVECs from a single donor were stimulated with VEGF. Baseline sprouting and overall responsiveness changed through the course of this experimental approach, affecting the absolute but not the relative sprouting response (**Fig. 6*A***). Moreover, variations in the experimental outcome were increased when comparing the VEGF-induced or inhibitor-affected sprout formation of HUVECs with comparable population doublings isolated from different donors (Fig. 6*B*).

Immortalized HUVECs constitute a stabilized cellular source for the spheroid-based angiogenesis assay

To improve the consistency of this assay, iHUVECs were tested as an alternate, stabilized, and homogenous endothelial cell source with enhanced cell culture capacity. Immunofluorescence analyses detected comparable abundance of endothelial cell markers such as VE-cadherin and Ang-2 in HUVECs and iHUVECs (**Fig. 7*A–D***and Supplemental Fig. 2), and both cell types showed comparable induction of capillary sprouts on stimulation with VEGF (Fig. 7*E*), as well as enhanced sprouting on inhibition of the γ-secretase, which is known to regulate tip cell formation by cleavage of Notch (Supplemental Fig. 3). However, when repeating the aforementioned screening experiment and comparing the means from 3 different HUVEC preparations (passage 2/3) with those from 3 iHUVEC sets (passages 10, 15, and 21), the variability in the HUVEC group was clearly greater (**Fig. 8**).

DISCUSSION

Nowadays, most *in vitro* studies focusing on the behavior of human vascular endothelial cells use HUVECs as the primary source of cultured cells, which were originally introduced by Jaffe *et al.* (22). Because of their availability, easy preparation, and remarkable growth capacity (23), this cell type forms the *in vitro* backbone of vascular research. However, they gradually lose their original phenotype if maintained in standard 2-dimensional cell culture (24), and their responses may be biased by a plethora of exogenous factors. As HUVECs are isolated from umbilical cords, their phenotype may be affected from fetal distress, pregnancy-associated diseases such as preeclampsia, and any medication of the donors. Additionally, they may be exposed to different anoxic or hypoxic or hypo- or hyperthermic episodes until and during preparation. Once in culture, their responses are greatly affected by culture conditions, cell density, proliferation, age, and HUVEC genotype. For instance, the $^{-786}$C/T single nucleotide polymorphism in the endothelial NO synthase (*NOS-3*) gene (25) contributes to a plethora of phenotype changes resulting from different *NOS-3* expression levels and activities (26). As 3-dimensional spheroid cell culture conditions have been shown to overcome some of these limitations, thus supporting homogenous HUVEC responses to individual stimuli, this study explored whether this extends to the sensitivity and reproducibility of the spheroid-based angiogenesis assay.

Our findings underline that this assay supports the formation of tip cells that precede the elongation of individual capillary-like sprouts originating from single spheroids. Their morphology and filopodia-like extensions are compatible with the original description of these cells (1).

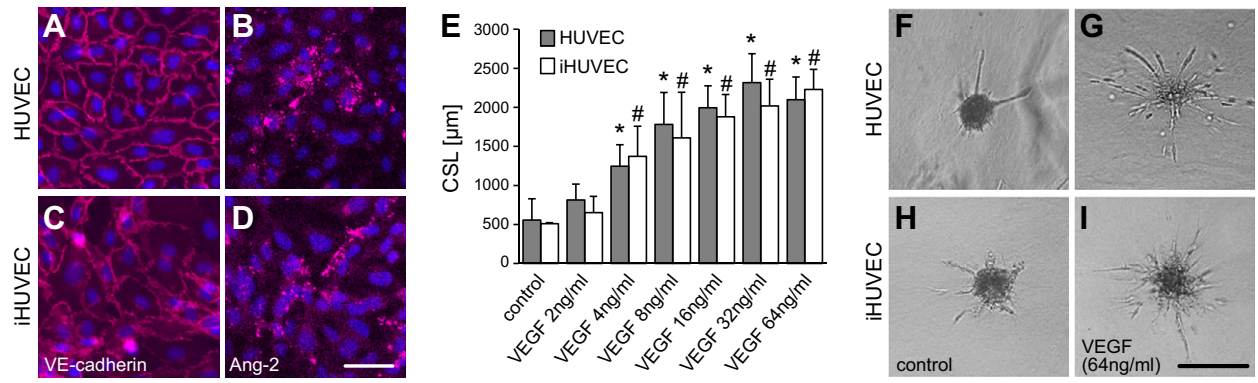

Figure 7. Characterization of iHUVECs and their usability for the spheroid-based angiogenesis assay. *A–D*) Endothelial cell markers (VE-cadherin and Ang-2) were detected in both HUVECs and iHUVECs. Scale bar, 50 μm. *E*) Collagen gel-embedded HUVEC and iHUVEC spheroids were stimulated with increasing doses of VEGF for 24 hours. $^{*}P < 0.05$ *vs.* control (HUVECs), $^{\#}P < 0.05$ *vs.* control (iHUVECs). *F–I*) Representative images of corresponding spheroids are shown. Scale bar, 200 μm. Mean CSL was calculated for 10 randomly selected spheroids per experimental group.

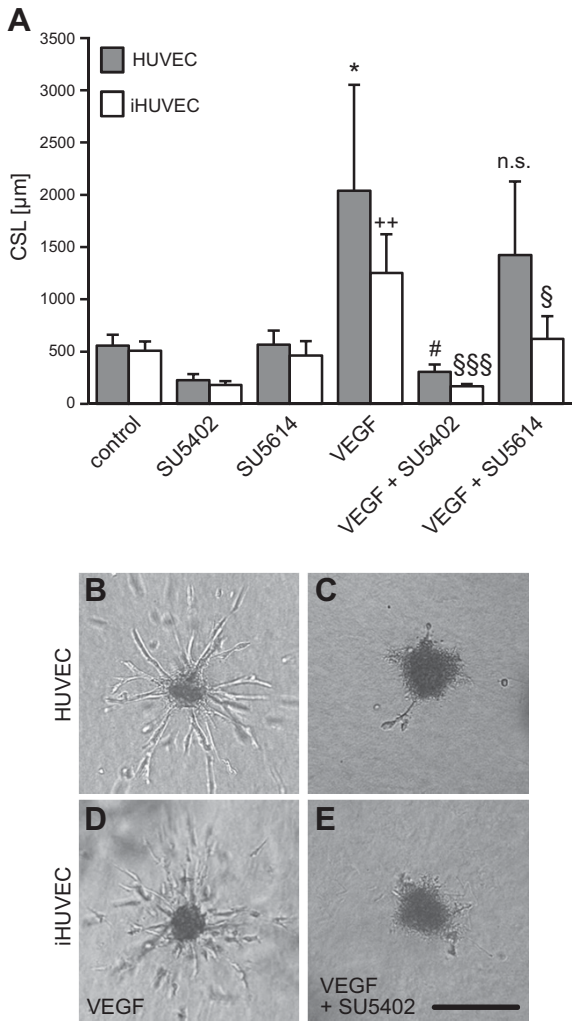

Figure 8. Comparing sprouting responses of HUVECs and iHUVECs. *A*) Angiogenic sprouting from spheroids generated from 3 different HUVEC preparations and iHUVECs of different passages was stimulated or inhibited (24 hours) by VEGF (25 ng/ml), SU5402 (10 μM), and SU5614 (10 μM), respectively. $^{*}P < 0.05$ *vs.* control (HUVECs), $^{\#}P < 0.05$ *vs.* VEGF (HUVECs), n.s., not significant *vs.* VEGF (HUVECs), $^{++}P < 0.01$ *vs.* control (iHUVECs), $^{\S\S\S}P < 0.001$ *vs.* VEGF (iHUVECs), $^{\S}P < 0.05$ *vs.* VEGF (iHUVECs). *B–E*) Representative images of corresponding spheroids are shown. Scale bar, 200 μm. Bars represent the mean CSL ± SD of 3 experiments determining 10 randomly selected spheroids per group and each experiment.

Furthermore, the genesis of the spheroid-derived tip cells was shown to be dependent on Wnt, that is, Delta-Notch signaling (18, 19). Likewise, we revealed the formation of elongated lumenized capillary structures as has been evidenced in sprouts originating from bovine aortic endothelial cell spheroids in earlier studies (10). Together with the generally high sensitivity of HUVEC spheroids to prototypic proangiogenic stimuli, these observations suggest that this assay is capable of physiologically mimicking relevant steps of the angiogenic cascade *in vitro*. Endothelial cells derived from adult blood vessels such as saphenous veins or aortae, on the other hand, may be less sensitive to corresponding angiogenic stimuli.

The overall outcome of spheroid-derived angiogenic sprouting is mostly influenced by the baseline activity, which is donor/preparation dependent and increases with the age of the cultured HUVECs. Although donor-dependent variability is an intrinsic problem that all models based on primary human cells have to deal with, age-mediated changes in endothelial cell properties may be compensated by using cells with comparable population doublings. Consequently, pro- and antiangiogenic responses were consistently evoked in collagen gel-embedded spheroids, but we realized differences between individual HUVEC preparations, which may limit the overall comparability of results obtained with this assay. Although the relative differences between results of individual approaches appeared to be marginally affected by these factors, they influenced the absolute range of angiogenic sprouting and may thus obscure subtle responses (*e.g.,* SU5614-mediated inhibition of VEGF-induced sprouting). Based on the relative robustness of the results produced by the spheroid-based angiogenesis assay, it has been used as a screening tool to delineate the pro- or antiangiogenic potential of therapeutic 880 compounds and to determine their functional IC_{50} values if applicable. Thereby, we delineated the antiangiogenic capacity of Perhexiline—an inhibitor of the mitochondrial enzyme carnitine palmitoyltransferase. In fact, appropriate *in vitro* screening systems covering the full range of angiogenic responses of human endothelial cells are scarce. Instead, for screening purposes, assay systems are frequently used that only reflect individual steps of the angiogenic cascade such as activation of distinct kinases (27), protein-protein interactions (28), or proliferation (29). Considering the limitations in transferring findings from animal experiments to the human setting and the difficulty in forecasting the relevance of individual molecules on complex signaling cascades, scrutinizing the impact of a specific compound within an artificial human assay system may have some notable advantages.

By using iHUVECs, allowing for extended propagation in cell culture and thus evading the need for HUVECs from multiple donors, we minimized the interexperimental variations. Although immortalization of human endothelial cells is often accompanied by impaired VEGF receptor expression, loss of the endothelial cell phenotype, or limited angiogenic responses (30–32), the iHUVECs used throughout this study were capable of forming capillary-like sprouts in response to VEGF stimulation up to passage 21. However, we more frequently observed formation of interrupted sprouts indicating that the functional phenotype of these cells is not fully comparable with that of HUVECs. In the long run, however, iHUVECs may bear an advantage for the consistency of larger *in vitro* angiogenesis screening approaches.

In a nutshell, this study underlined the high sensitivity, robustness, and cell type compatibility of the spheroid-based angiogenesis assay and its capacity for identifying pro- and antiangiogenic growth factors/drugs, as well as calculation of their functional IC_{50} values. Consequently, it may serve as a versatile and effective tool for future screening approaches focusing on human angiogenesis or specifically targeting tip cell formation. FJ

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Endothelial cell spheroids as a versatile tool to study angiogenesis *in vitro*

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Supplement I:

Summary of the screening results (drugs with no effect on VEGF-induced sprouting (++) were not shown)

Legend - HUVEC: VEGF-induced sprouting (baseline): ++; strong inhibition of VEGF-induced sprouting: 0, weak inhibition of VEGF-induced sprouting: +, no effect on VEGF-induced sprouting: ++, enhancement of VEGF-induced sprouting: +++

Legend - fibroblasts: baseline sprouting: ++, strong inhibition baseline sprouting: 0, weak inhibition of baseline sprouting: +, no effect on baseline sprouting: ++, enhancement of baseline sprouting: +++

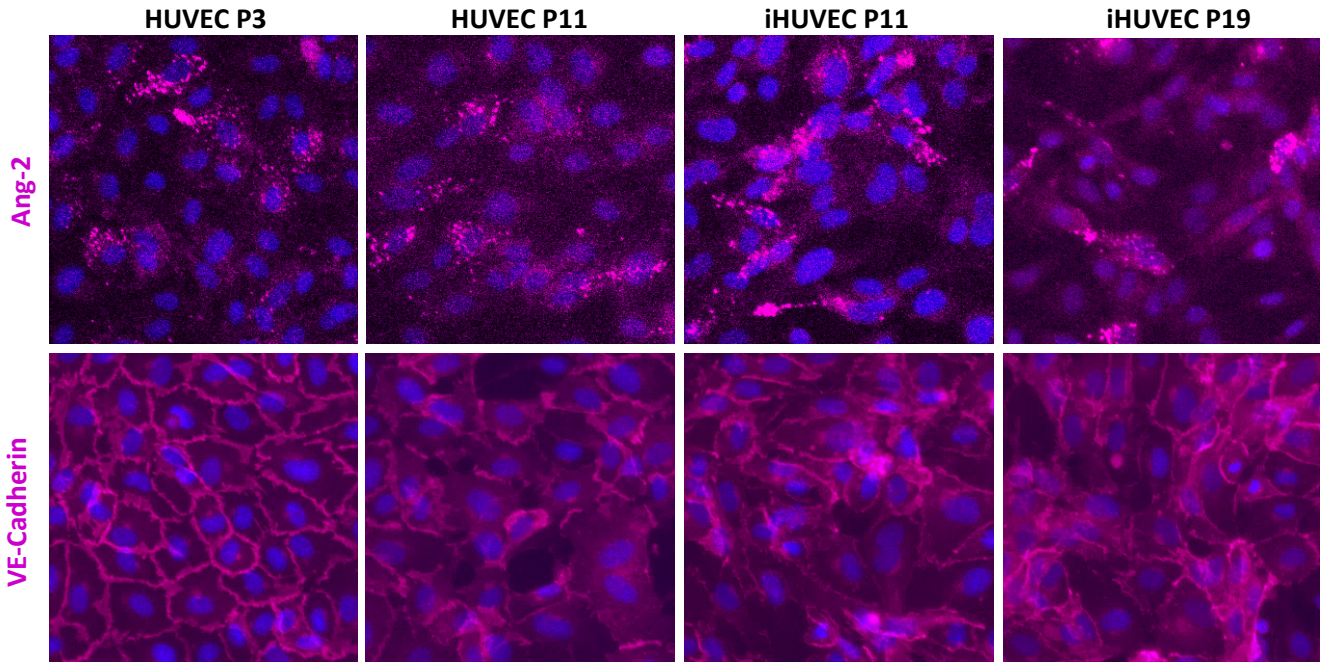
Chemical name	HUVEC sprouting	fibroblast sprouting
Acebutolol hydrochloride	+++	++
Aceclofenac	+	++
Albendazole	0	0
Amidopyrine	+	++
Anisomycin	0	0
Aposcopolamine	0	++
Ascorbic acid	++	
Astemizole	+++	+++
Betamethasone	+	++
Bisacodyl	0	+
Camptothecine (S,+)	0	+
Canrenoic acid potassium salt	+	++
Carmustine	+	+
Celestin blue	0	+ (0)
Cephaeline dihydrochl. heptah.	0	0
Chelidonine monohydrate (+)	0	0
Chlorpromazine hydrochloride	+++	+++
Chlortetracycline hydrochloride	+	++
Ciclopirox ethanolamine	+++	+
Clenbuterol hydrochloride	+	++
Clobetasol propionate	+	+
Colchicine	0	0
Cortisone	++	
Cotinine (-)	+	++
Cycloheximide	0	0
Cyproheptadine hydrochloride	+++	+++
Danazol	+++	++
Daunorubicin hydrochloride	0	++
Dexamethasone acetate	+	++
Digitoxigenin	0	0
Digoxin	0	0
Disulfiram	0	+
Doxorubicin hydrochloride	0	++
Dyclonine hydrochloride	+	+
Ebselen	+	+
Ellipticine	0	+
Emetine dihydrochloride	0	0
Erythromycin	+	++
Fenbendazole	0	0
Fludrocortisone	+	++
Flumethasone	+	++
Flutamide	+++	++
Fluvastatin sodium salt	+	+
formyl-(N)-deacetylcolchicine	0	0
Galanthamine hydrobromide	+	++
Gallamine triethiodide	+	++
Gemfibrozil	+	++
Griseofulvin	+	++
Haloprogin	0	0
Heptaminol hydrochloride	+	++
Hydrocortisone base	0	++
Indomethacin	+	++
Iproniazide phosphate	+	++
Ivermectin	+	++
Josamycin	+	+
Lanatoside C	0	0
Lisuride (S)(-)	+++	++
Lycorine hydrochloride	0	0
Mebendazole	0	0
Mechlorethamine hydrochloride	0	+
Mefexamide hydrochloride	+	++
Mefloquine hydrochloride	+++	+++
Menadione	0	0
Methylene blue	0	++
Methylprednisolone, 6-alpha	+	++
Mianserine hydrochloride	+++	+++
Miconazole	+++	++
Midecamycin	+	++
Minaprine dihydrochloride	+++	++
Minoxidil	+	++
Mometasone furoate	+	++
Mycophenolic acid	+	++
Niclosamide	0	0
Nocodazole	0	0
Oleandomycin phosphate	+	++
Paclitaxel	0	0
Pargyline hydrochloride	+	++
Parthenolide	0	0
Perhexiline maleate	+	++
Perphenazine	+++	++
Pheniramine maleate	+++	++
Piperlongumine	0	0
Piroxicam	+	++
Podophyllotoxin	0	0
Praziquantel	+	++
Prednisolone	+	++
Puromycin dihydrochloride	0	0
Pyrithione sodium salt	0	0
Rotenone	0	0
Scoulerin	0	++
Strophanthidin	0	0
Strophantine octahydrate	0	0
Syrosingopine	+++	+++
Terconazole	+	+
Thimerosal	0	0
Thioguanosine	+	++
Thioridazine hydrochloride	+++	+++
Thiourea, 1-phenyl-3-(2-thiazolyl)	+	+
Tiaprofenic acid	+	++
Tolazoline hydrochloride	+++	+++
Tolbutamide	+	++
Tomatidine	+++	++
Tranylcypromine hydrochloride	+	++
Trimetazidine dihydrochloride	+++	++
Zidovudine, AZT	+	+

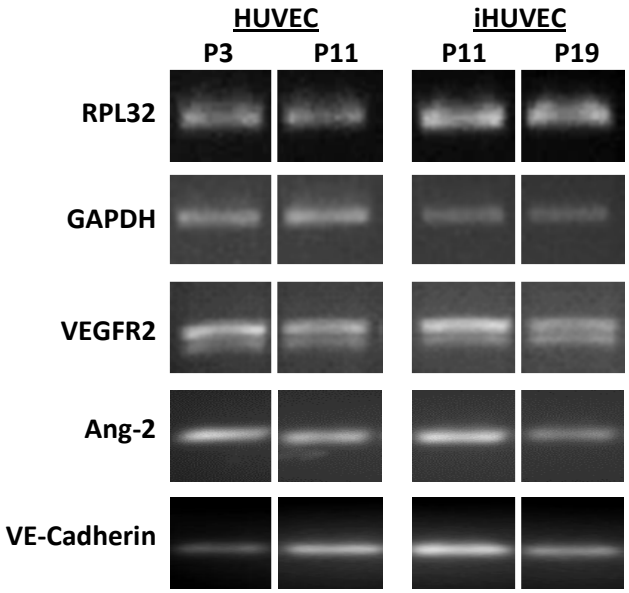
Supplement II:

Comparison of endothelial cell marker gene expression in HUVEC and iHUVEC

HUVEC and iHUVEC have been analyzed upon different culture times by immunofluorescence analyses. While the abundance of Angiopoietin-2 (Ang-2) appeared to remain on a low level in both cell types, the regular VE-Cadherin staining pattern disappeared in HUVEC over time (nuclei were visualized by DAPI (blue) staining).

PCR analyses of these cells indicated that the expression of VEGF receptor 2 (VEGFR2) and Ang-2 slightly decreases over time while that of VE-Cadherin increases in HUVEC and decreases in iHUVEC (mRNA expression of the housekeeping genes RPL32 (ribosomal protein L32) as well as GAPDH were utilized as reference).





Supplement III:

Formation of sprouts is induced upon γ-secretase inhibition

PCR analyses verified the mRNA expression of Dll4 and Notch4 in both HUVEC and iHUVEC (A). To manipulate tip cell formation, spheroids were treated with DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) – an inhibitor of the γ-secretase. This enzyme controls tip cell formation by cleaving Notch in endothelial cells while its inhibition by DAPT accelerates tip cell formation (Hellström et al., Nature 445: 776-780, 2007). Likewise, tip cell and eventual sprout formation from HUVEC (B) and iHUVEC (C) spheroids was enhanced upon stimulation with DAPT (B and C, *p<0.05 25 µM DAPT vs. appropriate DMSO control; #p<0.05 50 µM DAPT vs. appropriate DMSO control; bars represent the mean cumulative sprout length (CSL) ± SD of 10 randomly selected spheroids per experimental group).