ONLINE SUPPLEMENT

Tie2-integrin signaling balance determines Angiopoietin-2 functions in human endothelial cells and mouse angiogenesis

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Felcht, et al., Supplemental Figure 1

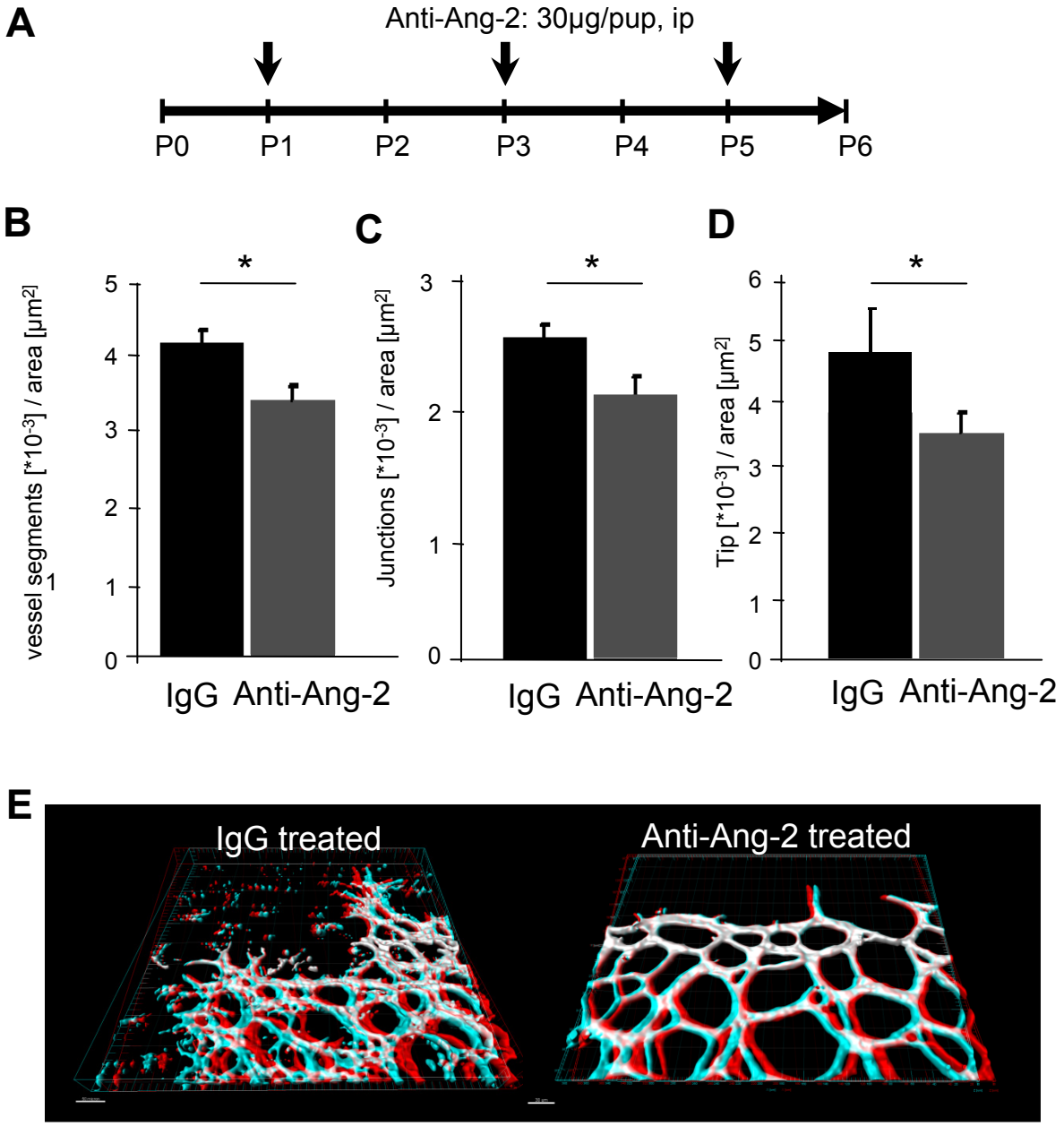


Figure 1
Effect of Ang-2 blockade on postnatal retinal angiogenesis. **(A)** Schematic protocol of the injection of systemic anti-Ang-2 antibody treatment of mouse pups. Newborn mice were intraperitoneally injected (i.p.) with 30µg/pup at postnatal days 1, 3 and 5 and sacrificed at day 6 for eye enucleation. The observed reduction of junctional branchpoints (Figure 1G), number of vessel segments (Figure 1H) and tip cells (Figure 1K) was independent of the reduction of the total vessel area (Figure 1B). The total number of vessel segments **(B)**, of junctional branchpoints **(C)** and tip cells **(D)** was calculated with Fiji software. $P<0.05$. **(E)** The retinas were visualized with lectin-FITC and images were acquired by confocal microcopy (Leica SP5). A 3D computer model of IgG treated vs. anti-Ang-2 treated retinas was reconstructed with Amira software. Anaglyphic 3D glasses are required for 3D visualization.

Felcht, et al., Supplemental Figure 2

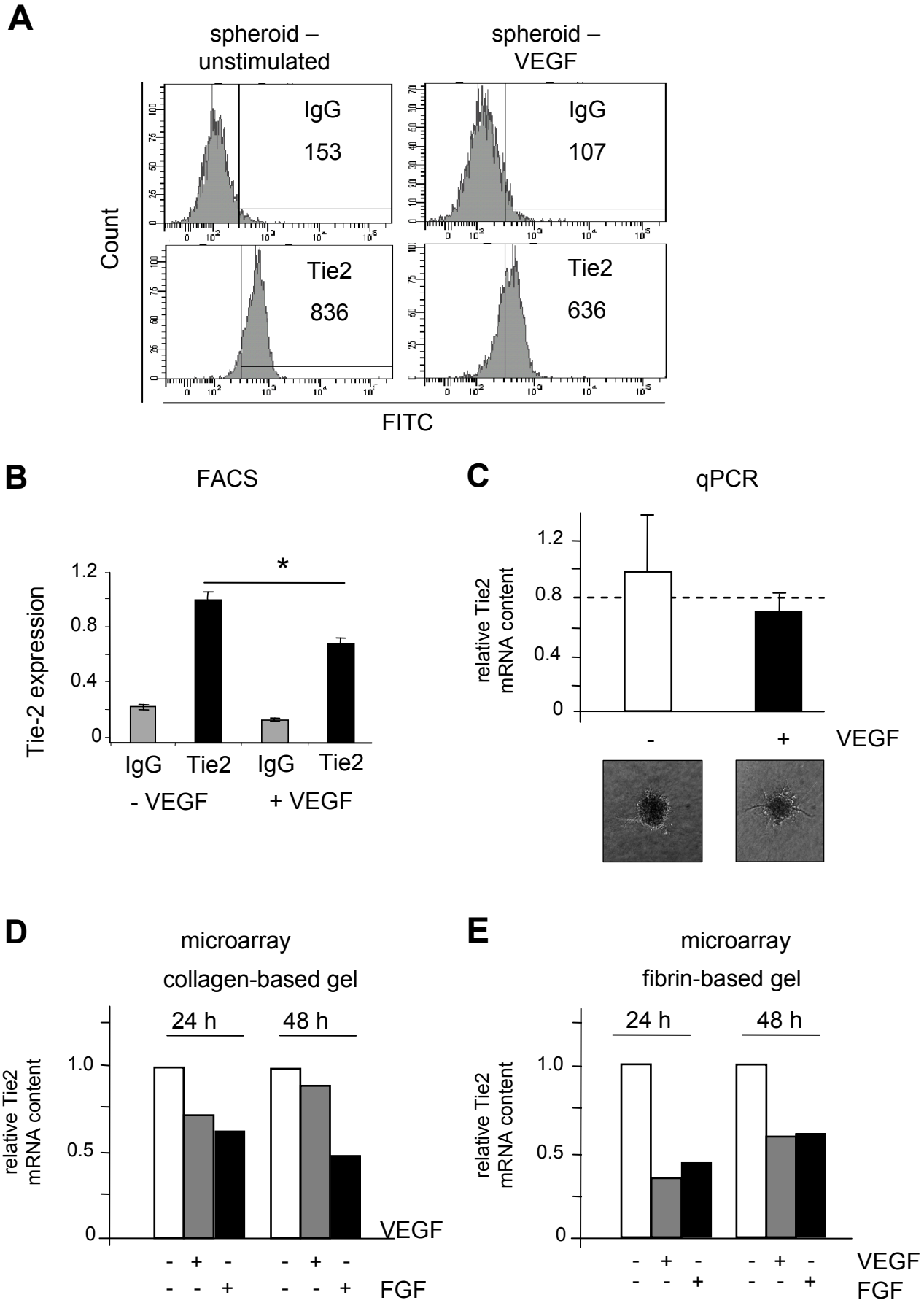


Figure 2
Transcriptional regulation of Tie2 expression in different cellular models of endothelial cell activation and angiogenesis **(A, B)** Quantitative assessment of mean Tie2 expression in the EC spheroid sprouting angiogenesis assay. Cells were allowed to sprout for 48h in the presence or absence of VEGF. Cells were harvested and Tie2 expression was analyzed by FACS. The upper panel shows one representative experiment. Mean Tie2 expression is indicated as mean fluorescence intensity. The bottom panel shows the quantitative analysis of five independent experiments. VEGF stimulation significantly downregulated EC Tie2 expression during sprouting angiogenesis (*; $p<0.05$). **(C)** Quantitative assessment of relative Tie2 mRNA content during sprouting angiogenesis. Sprouting of HUVEC cells was induced by VEGF stimulation using the spheroid angiogenesis assay. Spheroids were digested and studied by qPCR for relative Tie2 expression. PDH was used as the house-keeping gene. **(D, E)** Quantitative assessments of Tie2 mRNA content during sprouting angiogenesis using different extracellular matrices. HUVEC spheroids were implanted into collagen or fibrin gels. Sprouting was induced with VEGF or FGF for 24h and 48h. Microarray analysis was performed and relative Tie2 mRNA was compared under sprouting and control conditions.

Felcht, et al., Supplemental Figure 3

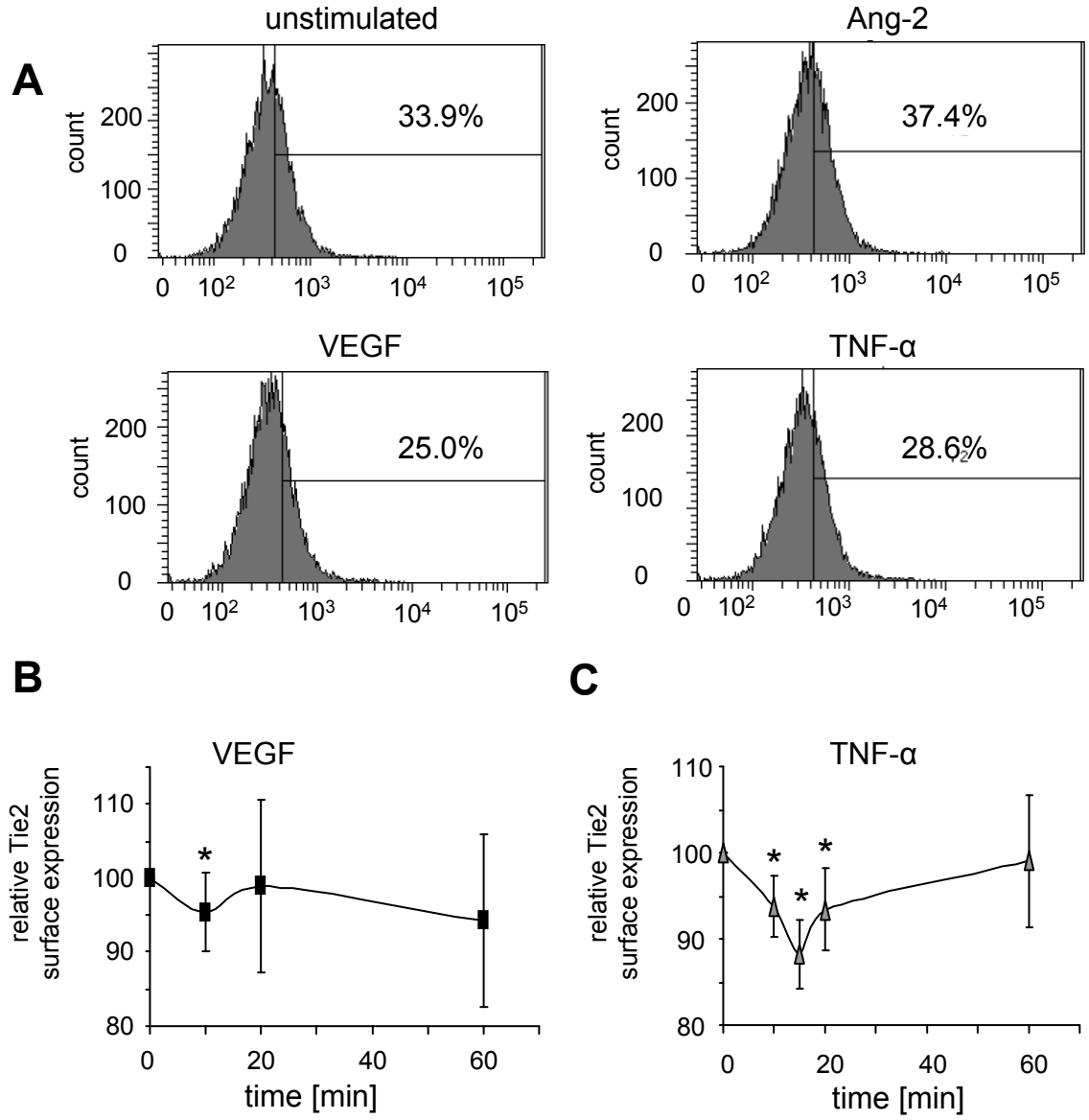
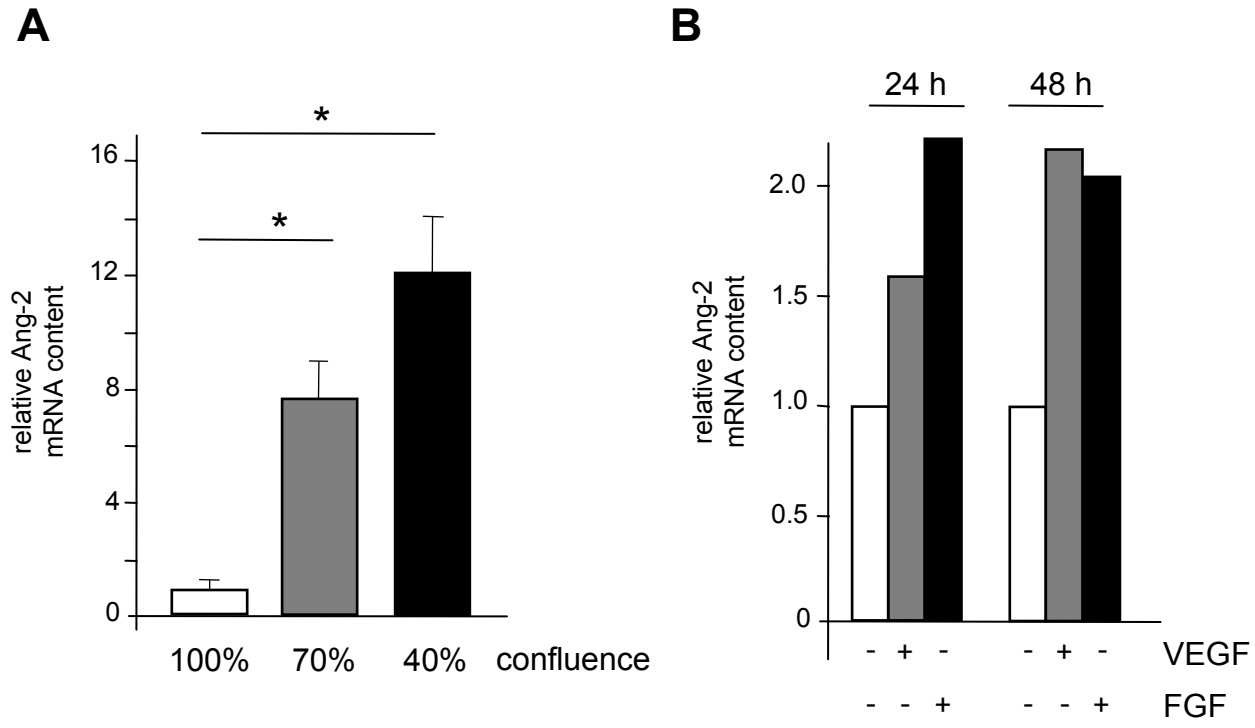


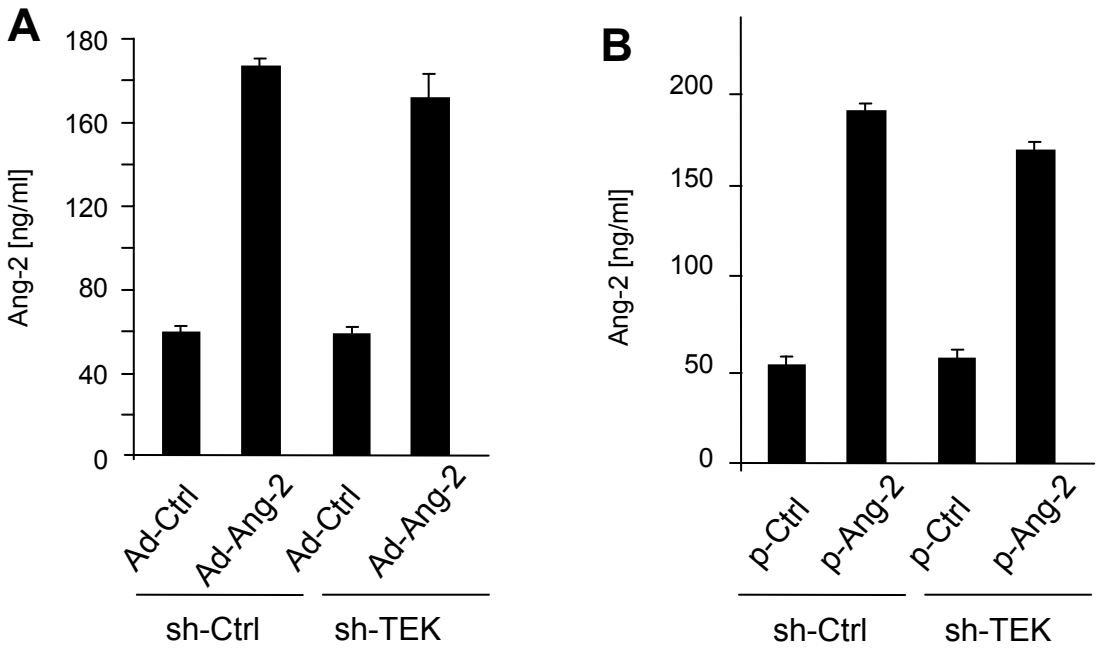
Figure 3
Transcriptional regulation of Tie2 expression in different endothelial cell culture models. **(A-C)** Quantitative assessment of mean Tie2 surface expression in monolayers of EC stimulated with different angiogenic cytokines (Ang-2, VEGF and TNF-α) for different periods of time up to 60min. Cells were harvested and Tie2 expression was analyzed by FACS. The upper panel shows one representative experiment of 3 independent experiments. VEGF **(B)** and TNF-α **(C)** stimulation significantly downregulated EC Tie2 surface expression (*; $p<0.05$).

Felcht, et al., Supplement Figure 4



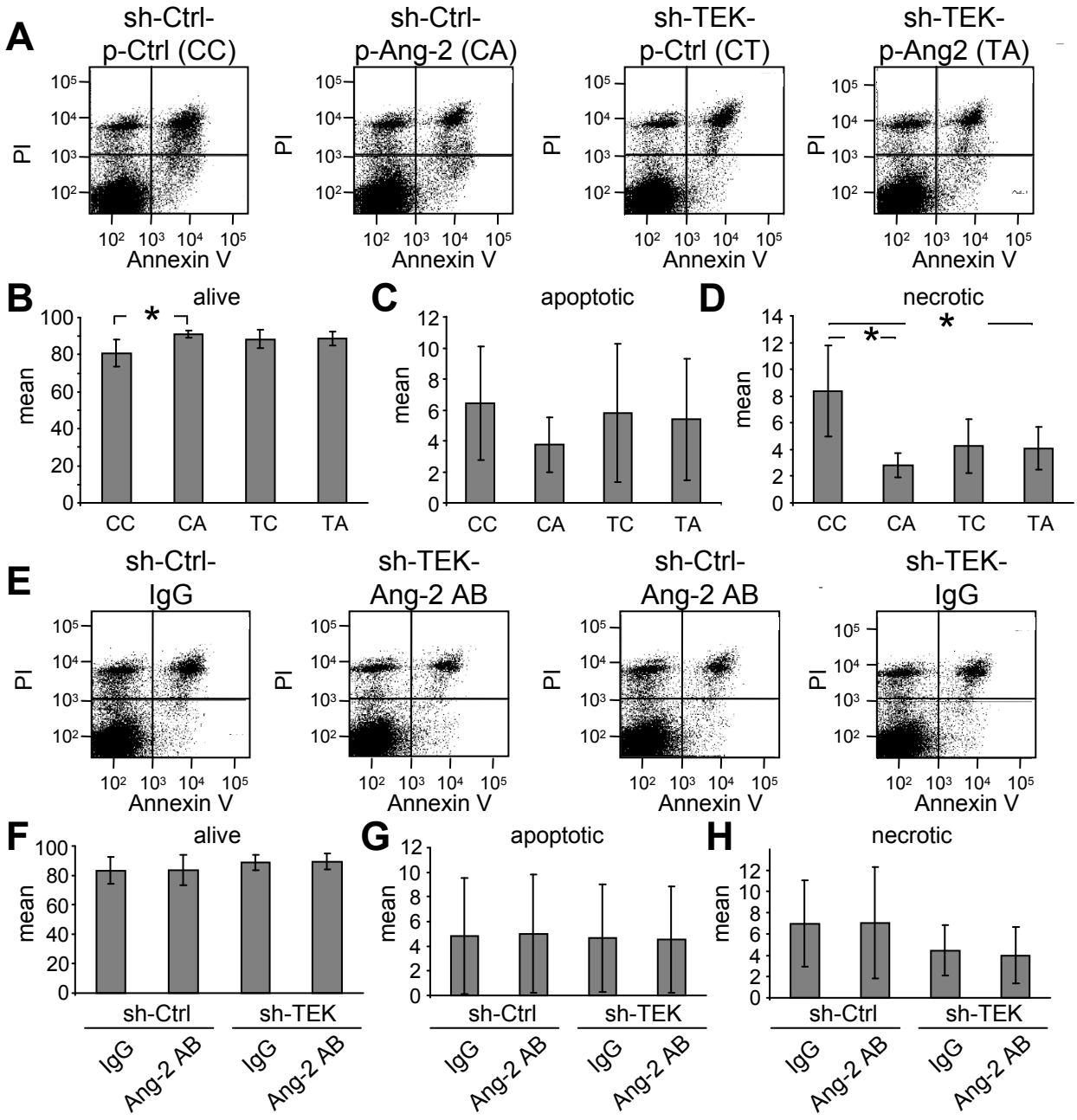
Supplement Figure 4
Transcriptional upregulation of Ang-2 expression in angiogenically activated endothelial cells. **(A)** Quantitative analysis of relative Ang-2 mRNA content in confluent and subconfluent monolayers of HUVEC. HUVEC were grown at different degrees of confluence as indicated, harvested and analyzed by qPCR. PDH was used as reference house keeping gene. Ang-2 expression in subconfluent HUVEC was strongly upregulated compared to confluent cells (*, $p<0.05$; n=3). **(B)** Quantitative analysis of endothelial Ang-2 mRNA content in the *in vitro* spheroid angiogenesis assay. Spheroids were embedded in a collagen matrix and allowed to form capillary sprouts induced by VEGF or bFGF for 24h and 48h, respectively. Microarray studies were performed and relative Ang-2 expression was compared between VEGF or FGF treated and untreated control cells.

Felcht, et al., Supplemental Figure 5



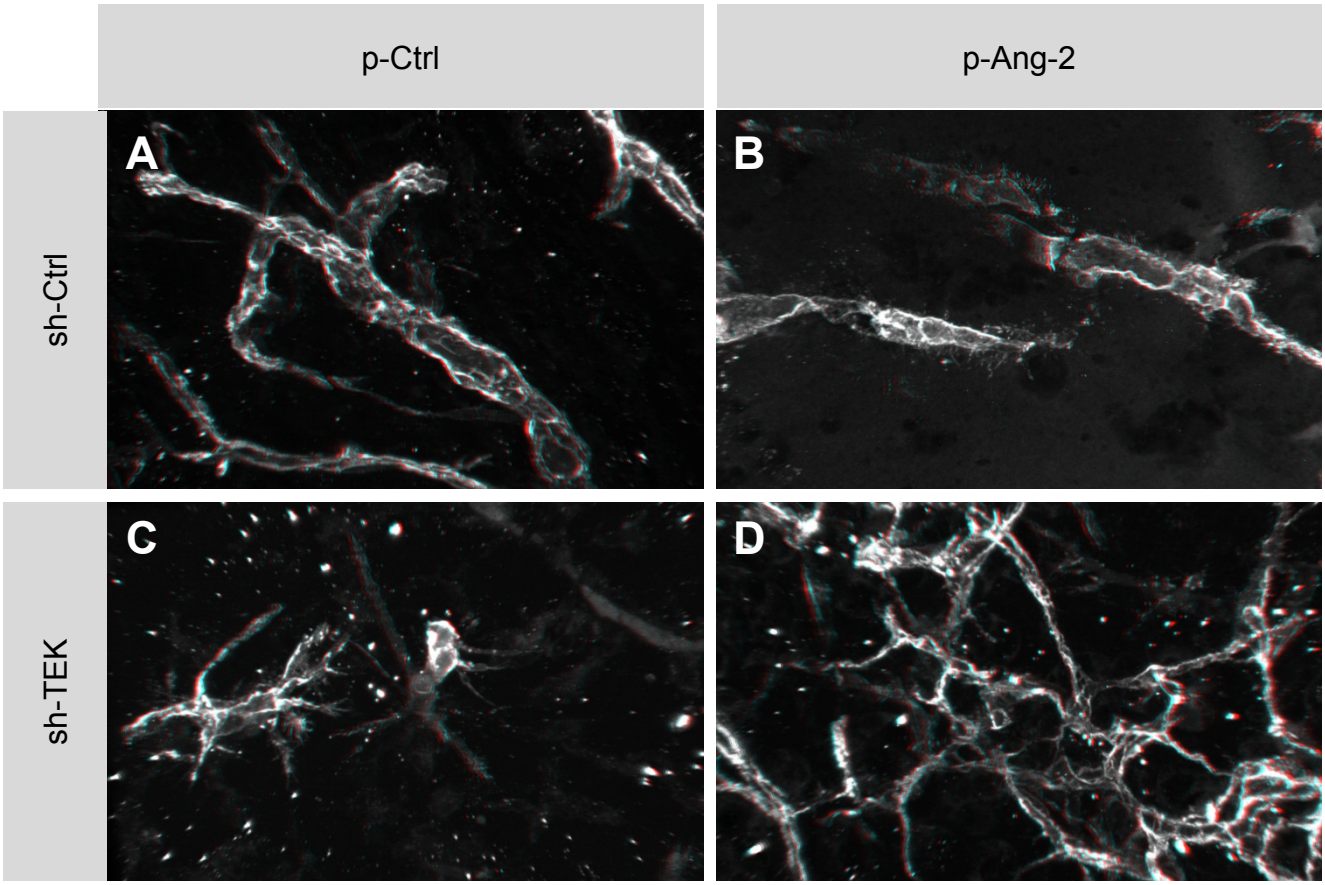
Supplemental Figure 5
Control Ang-2 protein measurements in supernatants of stably Tie2^{low} and Ang-2^{high} expressing EC. **(A, B)** EC were lentivirally transduced with sh-RNA silencing Tie2 (monitored by Western blot analyses (see Figure 3F or FACS analyses [Supplement Figure 10D] for representative characterization) and adenovirally overexpressing Ang-2 (Ad-Ang-2) **(A)** or lentivirally overexpressing Ang-2 (p-Ang-2) **(B)**. Ang-2 protein was measured by ELISA in the supernatants of the cells.

Felcht, et al., Supplemental Figure 6



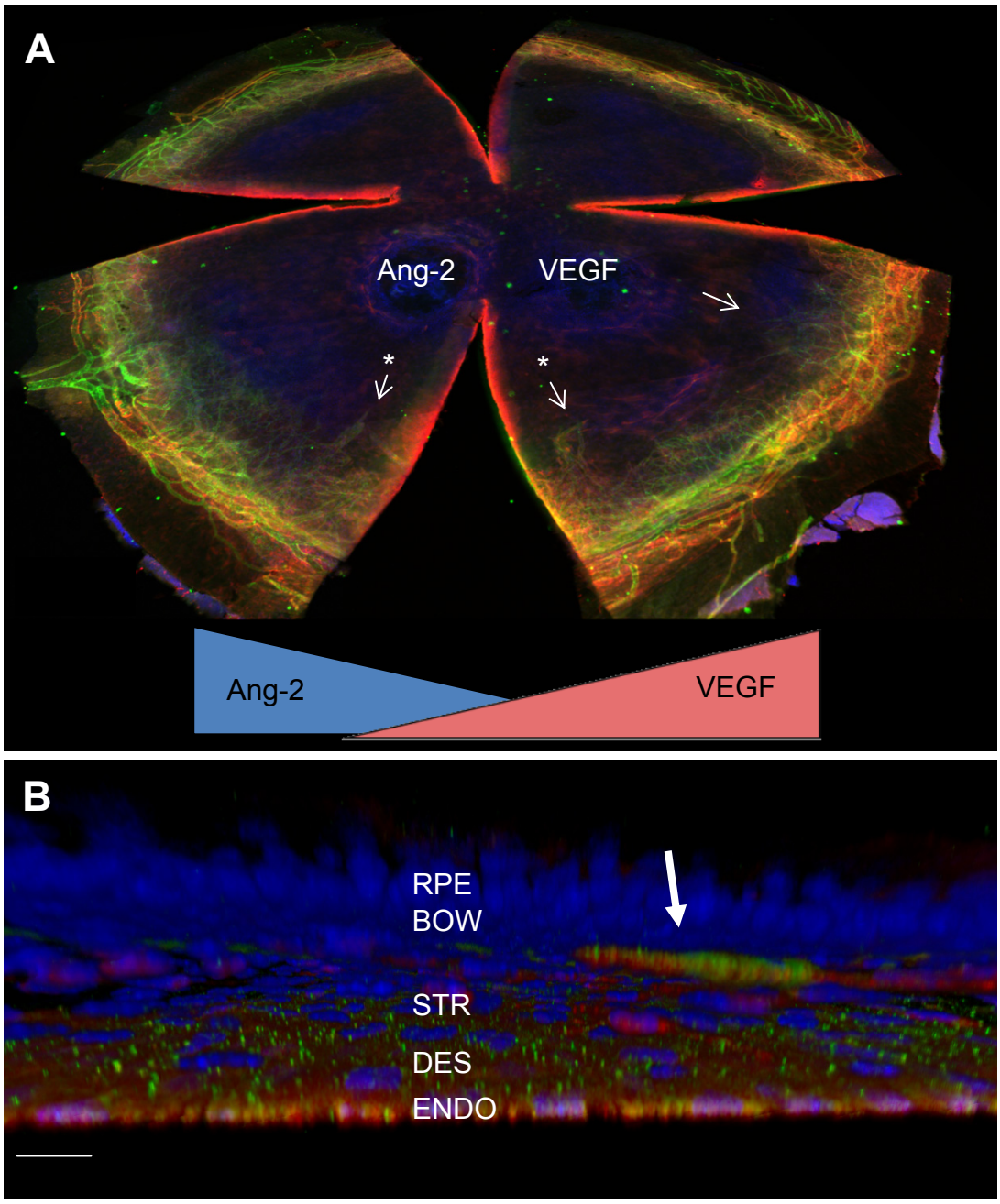
Supplemental Figure 6

Ang-2 upregulation protects EC from necrosis independently of Tie2 expression. **(A-D)** Control lentivirally transduced EC (sh-Ctrl), lentivirally Tie2-silenced EC (sh-TEK) as well as Ang-2 control (p-Ctrl) EC and Ang-2 overexpressing EC (p-Ang-2) were studied by FACS to assess living EC, apoptotic EC and necrotic EC. The cells were examined with the Annexin V apoptosis detection kit APC after they had been gated for GFP positivity. Ang-2 overexpression inhibited Tie2-independent necrosis of EC but did not significantly change apoptosis. **(E-H)** Ang-2 antibody treatment was compared to IgG antibody control treatment of Tie2 expressing EC (sh-Ctrl EC) and Tie2 silenced EC (sh-TEK). The Ang-2 antibody did not induce apoptosis or necrosis.



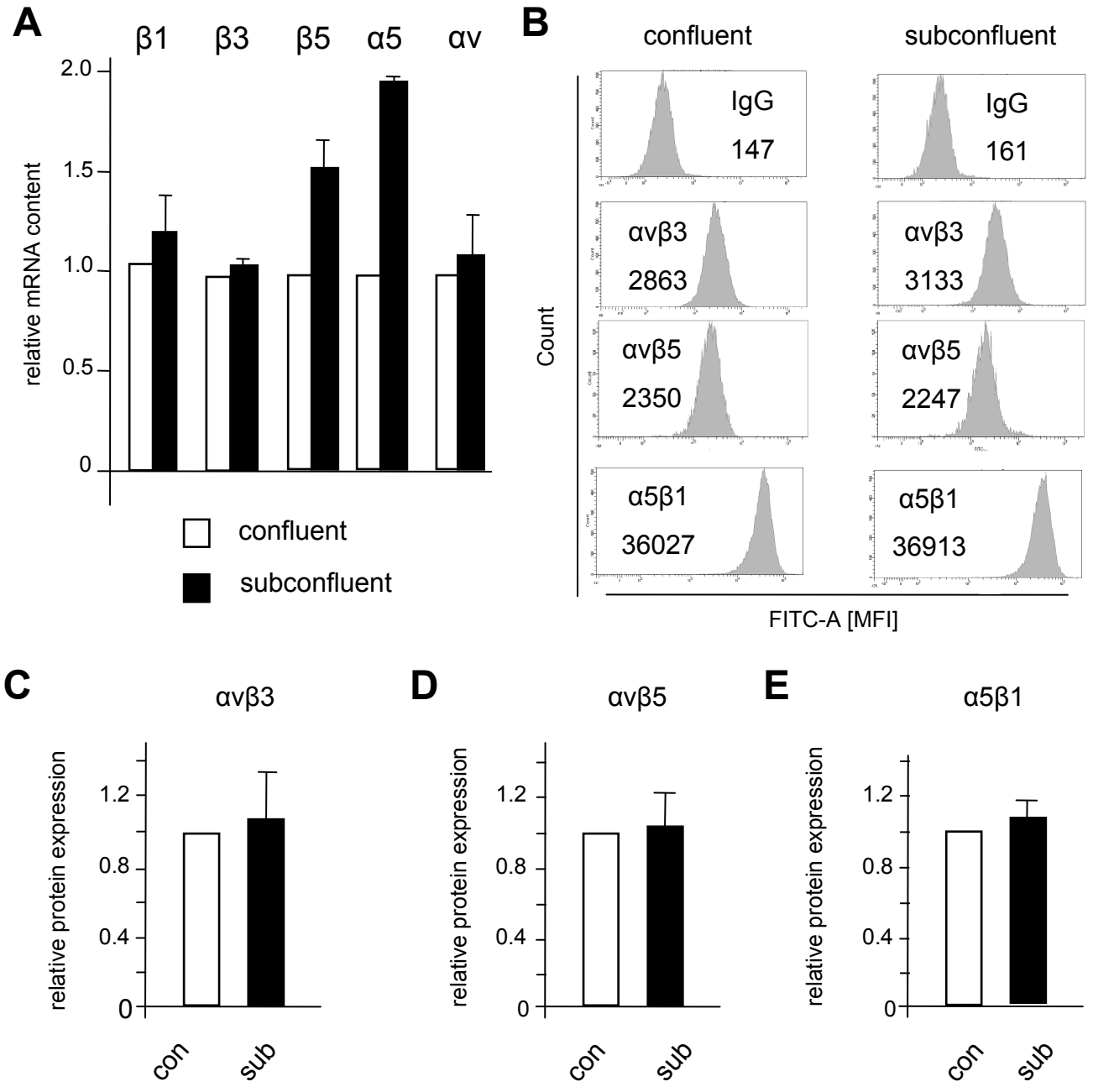
Supplemental Figure 7
Ang-2 induces tip cell rich vascular hypersprouting of Tie2 negative EC. **(A-D)** Vessel network formation of control transduced (sh-Ctrl) and Tie2 silenced EC (sh-TEK) doubled transduced with control lentivirus (p-Ctrl) or lentiviral Ang-2 (p-Ang-2) in the Matrigel xenografting assay was performed as described (Ref. 1, 2). Sections [50µm] were antibody-stained for CD31, followed by confocal microscopy and visualisation with Amira software. Anaglyphic 3D glasses are required for 3D visualization.

Felcht, et al., Supplemental Figure 8



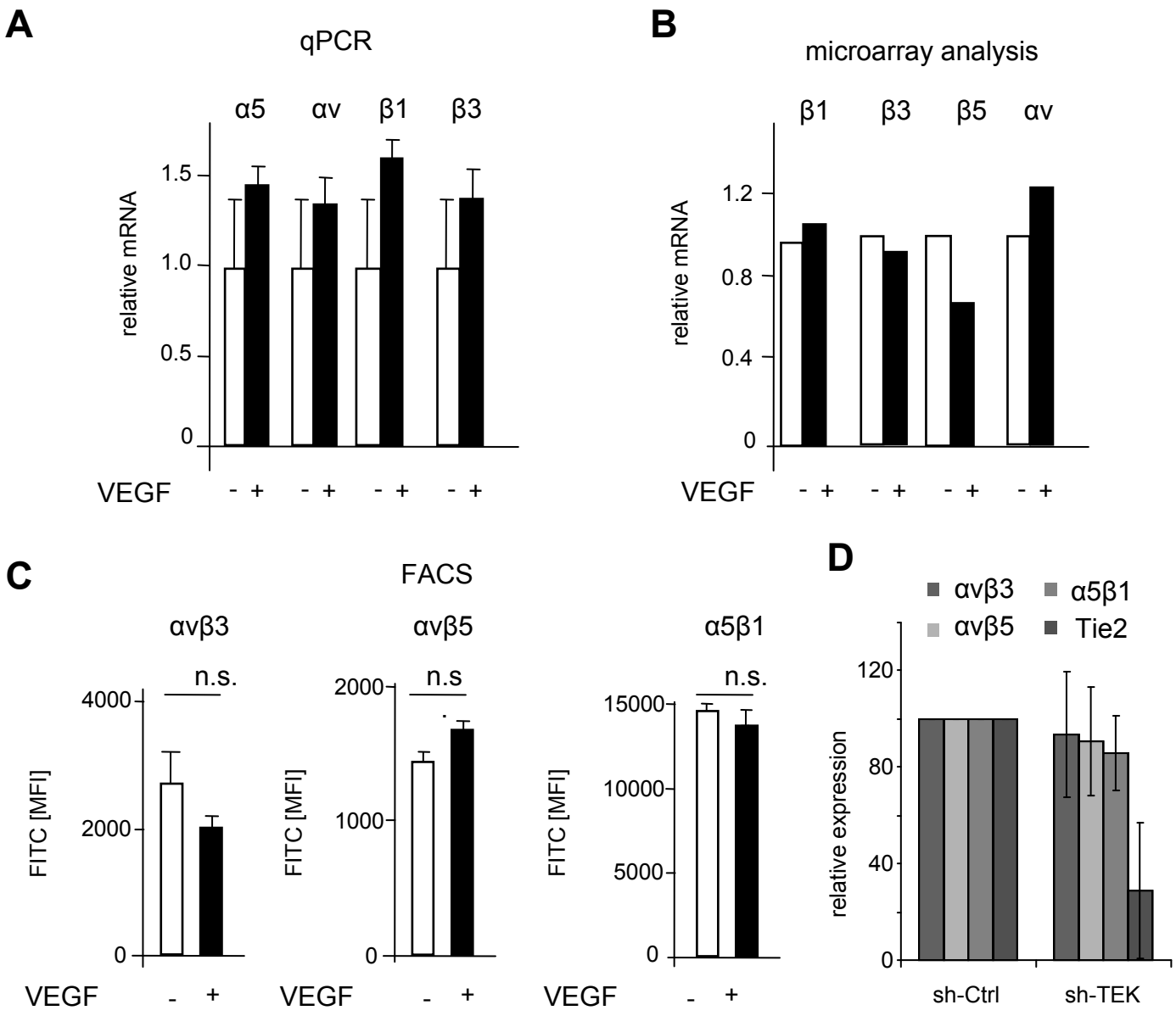
Supplemental Figure 8
Ang-2 induces vascular sprouting and FAK phosphoryaltion at Tyrosine 397 in tip cells in the cornea pocket angiogenesis assay. **(A)** The cornea pocket assay was performed with a double implantation of VEGF and Ang-2, followed by stimulation for 6 days, whole mount staining for CD31 and p-FAK[Tyr397]. Specimen were visualized by confocal imaging with tilf function. Higher magnifications with z-stack functions were taken from the VEGF stimulated area (arrows, right side of the dissected cornea) and of VEGF and Ang-2 stimulated areas (arrows with stars; left side of the dissected cornea). Pictures were visualized with Imaris 7.2.3.; Scale bar 400µm**. (B)** The cornea pocket assay was performed with VEGF and Ang-2 stimulation. CD31 and p-FAK[Tyr397] were stained and visualised by confocal imaging. Endothelial cells in the cornea (ENDO) strongly expressed p-FAK[Tyr397]. RPE (retinal pigment epithelium); BOW (Bowman's membrane); STR (stroma); DES (Descemet's membrane); ENDO (Endothelium). The arrow indicates a sprouting vessel which expresses p-FAK[Tyr397]. Scale bar 70µm. Supplement Video 3 gives a representative 3D impression of the dissected cornea.

Felcht, et al., Supplemental Figure 9



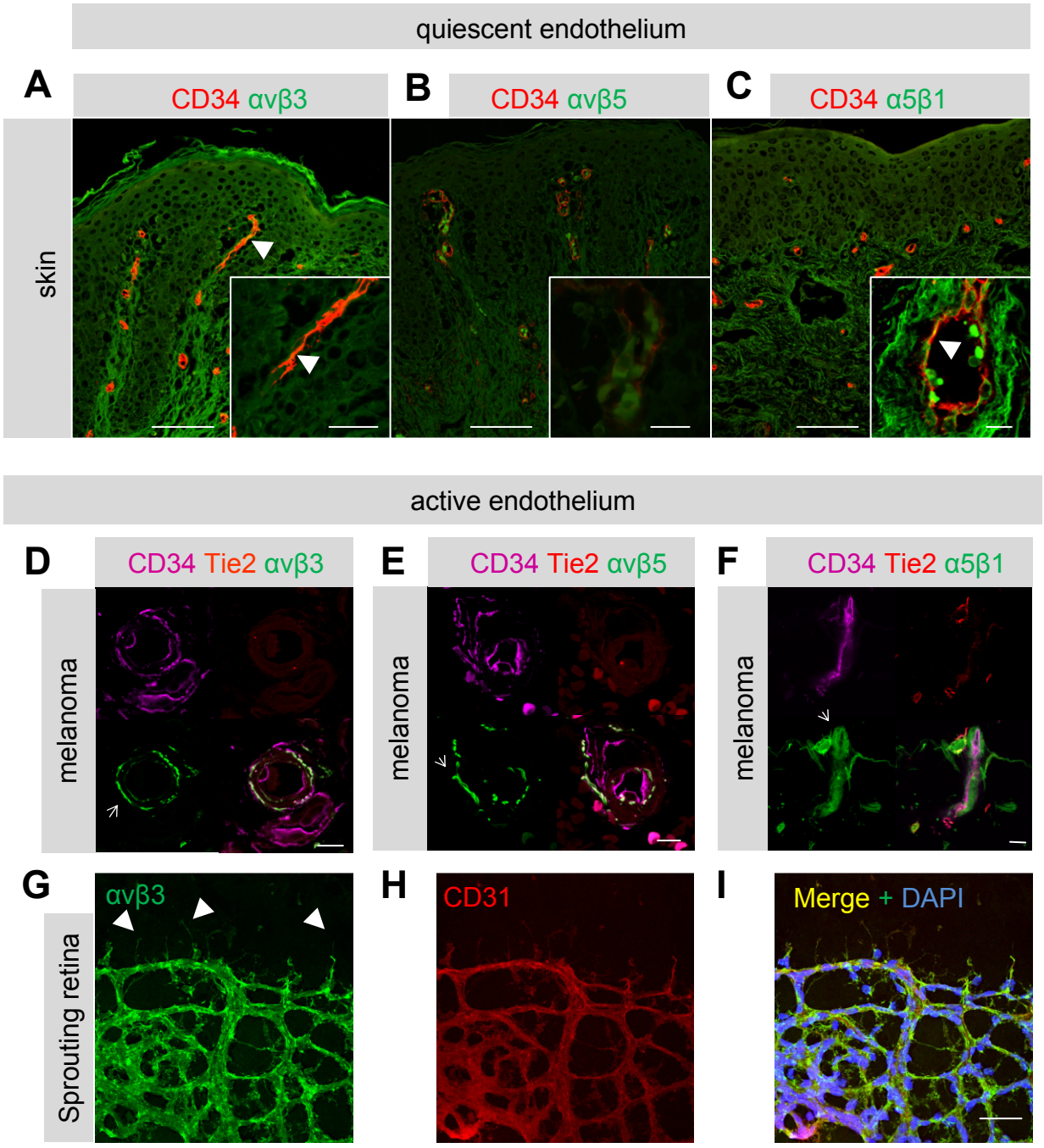
Supplementa Figure 9
Expression of the integrins αvβ3, αvβ5 and α5β3 in confluent and subconfluent EC. **(A)** Analysis of relative mRNA content of the integrin chains β1, β3, ß5, α5 and αv in confluent and subconfluent HUVEC monolayers. Confluent and subconfluent HUVEC were harvested and integrin mRNA content was analyzed by qPCR. PDH expression was used as the reference house keeping gene. **(B-E)** Analysis of angiogenic integrin protein expression in confluent and subconfluent HUVEC monolayers. Confluent and subconfluent HUVEC were harvested and studied by FACS analysis for their αvβ3, αvβ5 and α5β1 integrin heterodimer surface presentation. The relative expression of the integrin heterodimers αvβ3 **(C),** αvβ5 **(D)** and α5β1 **(E)** are shown as mean fluorescence intensity.

Felcht, et al., Supplemental Figure 10



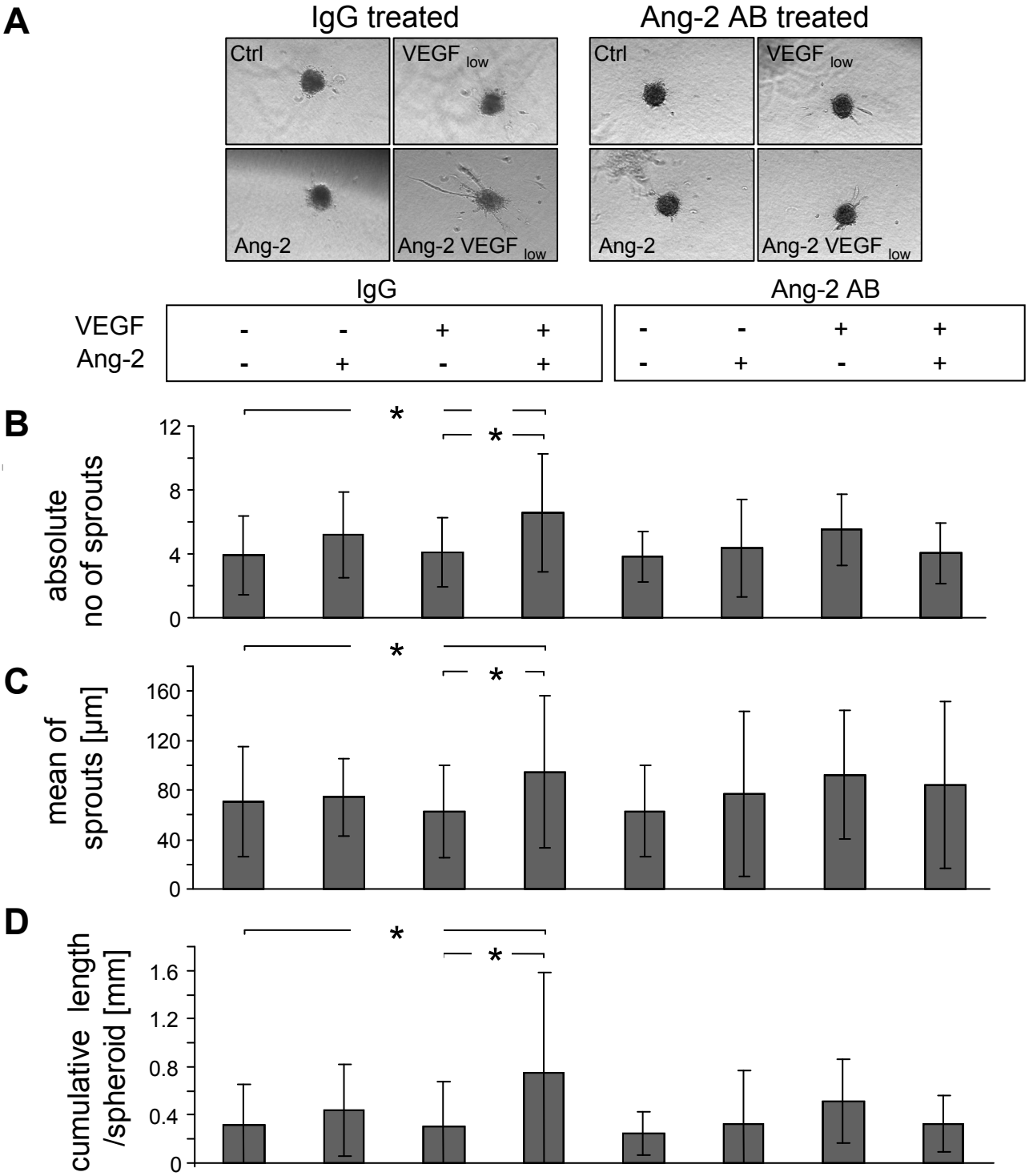
Supplemental Figure 10
Expression of αvβ3, αvβ5 and α5β1 Integrins in endothelial cells during sprouting angiogenesis. **(A, B)** Quantitative analysis of α5, αv, β1, β3 and β5 integrin mRNA expression by qPCR **(A)** and microarray analysis **(B)** in the spheroid sprouting angiogenesis assay. Spheroids were allowed to sprout for 48h, mRNA was isolated and relative mRNA content was analyzed by qPCR or microarray analysis, respectively. **(C)** FACS analysis of αvβ3, αvβ5 and α5β1 expression of EC harvested from the spheroid angiogenesis assay after 48h of sprouting angiogenesis (n.s. = not significant; n=4; student t-test). **(D)** Confluent EC monolayers with silenced Tie2 expression (sh-TEK) were studied by FACS analyses for integrin expression as well as Tie2 surface presentation.

Felcht, et al., Supplemental Figure 11



Supplemental Figure 11
Comparative analysis of αvβ3, αvβ5 and α5β1 integrin expression by quiescent, resting and angiogenic EC. Expression of the integrins was analyzed in the adult skin **(A-C)**, and in human melanoma samples **(D-F)** as well as the angiogenic postnatal retina **(G-I)**.The analyzed integrins are weakly to barely detectable in quiescent EC **(A-C)** and prominently upregulated in angiogenic EC **(D-I)**. Samples were double-stained with the indicated integrins and CD34 or triple stained for the indicated integrin and CD34 as well as Tie2. Whole mount retinas were stained with αvβ3 antibody and APC-labeled CD31. The integrin was visualized with Alexa488; DAPI was costained with the secondary antibody. Negative controls were performed by omitting the first antibody. Arrowheads indicate integrin-positive double labeling with the respective EC double staining marker (CD34). Bar graphs: 75µm (A-C overview); 50µm (G-I); 25µm A, C zoom, D-F; 10µm B zoom, L, O). Pseudocolors were used for visualization.

Felcht, et al., Supplemental Figure 12



Supplemental Figure 12

Ang-2 antibody treatment blocks Ang-2-induced sprouting angiogenesis. **(A)** Representative images of different experimental groups of sprouting EC in the spheroid angiogenesis assay are shown (unstimulated; Ang-2 stimulated; subcritical dose of VEGF stimulated (VEGF low); Ang-2 and subcritical dose of VEGF (VEGF low) stimulated under IgG treatment or Ang-2 neutralizing antibody treatment). **(B-D)** Quantitative assessment of the absolute number of vascular sprouts **(B)**, mean number of sprouts **(C)**, and cumulative sprout length **(D)** under IgG treatment or Ang-2 neutralizing antibody treatment. The summary of 3 independent experiments is shown 10 spheroids per experiment, S.D. ($p<0.05$).

Supplemental Table 1
Parameters for automated cell tracking*

Parameter	Value
Radius	6
Cutoff	0
Percentile	1
Link Range	3
Displacement	5

* the indicated parameters were included in the calculation of single-cell-dispersed cell migration.

Supplemental Experimental Procedures

Materials Recombinant Angiopoietin-2 (Ang-2), biotinylated recombinant Ang-2, recombinant soluble Tie2 receptor, recombinant VEGF and bovine fibronectin were purchased from R&D Systems. FITC conjugated Lectin from *Bandeiraea simplicifolia* was purchased from Sigma-Aldrich. The integrins αvβ3, αvβ5, and α5β1 were used from Millipore. The following antibodies were used: Mouse anti-αv (FB12), mouse anti-α2 (P1E6), mouse anti-α5, mouse anti-β3, mouse anti-β5, mouse anti-αvβ3 (LM609), mouse anti-αvβ5 (15F11) and mouse anti-α5β1 (JBS5) from Millipore/Chemicon, anti-CD31 (M0823) from R&D Systems, rat anti-CD34 (QBEnd10) from NovoCastra, mouse anti-Tie2 (Ab33) from Upstate, rabbit anti-Ang-2 from Abcam, rabbit anti-FAK [Tyr397] from Biosource, goat anti-β1, rabbit anti-β1, rabbit anti-FAK from Santa Cruz and mouse anti-Rac1 from BD Bioscience. Antibodies against the active β1 integrin subforms are commercially available from BDPharmingen (9EGF Cat. No.: 553715 and HUTS: Cat. No. 556047) (3, 4). IgG control was used from Jackson ImmunoResearch (Cat. 009-000-008). Annexin-V-FLUOS staining kit (Roche; Cat. No 11858777001) and Annexin V apoptosis detection kit APC (eBioscience; Cat. No 88-8007).

Cell culture Human umbilical vein endothelial cells (HUVEC), endothelial-cell growth medium (ECGM), endothelial-cell basal medium (EBM) and corresponding supplements were purchased from Promocell. Cells were cultured at 37°C, 5% CO2 in the appropriate growth medium containing 10% fetal calf serum (FCS) (Invitrogen). HUVEC were used between passages 2 and 5.

Lentiviral and/or adenoviral transduction For lentiviral transduction, 10 infectious units per cell were used. We transduced either with sh-RNA against Tie2 p-RNA against Ang-2 or with a control vector coding for GFP. Cells were plated in ECGM complete medium on cell culture dishes and allowed to adhere for 4h. Virus was added and cells were incubated for 24h. Fresh ECGM complete medium was added. Transduction efficacy was controlled by microscopic observation of GFP-positive cells or by Western blot for Tie2 or FACS analyses for Tie2 or by ELISA for Ang-2 overexpression.

For adenoviral transduction, 200 infectious units per cell were used. Cells were transduced either with an Ang-2 overexpressing vector or control vector. Cells were plated in ECGM complete medium in cell culture dishes and allowed to adhere for 4h. Virus was added and cells were incubated for 12h. Fresh ECGM complete medium was added. Cells were monitored for transduction efficacy by Ang-2 ELISA (R&D Systems). For co-transfected cells, these two protocols were used in tandem.

Automated cell tracking Raw image series were transformed to 8-bit images and then enhanced by ImageJ's built-in automated contrast enhancement. Tracking was done with the ImageJ plug-in ParticleTracker. Standard parameters were chosen within the range documented in Supplemental Table 1 to guarantee sensitive and specific tracking of cells and to exclude signals from cellular debris or tracks that intermingle two cells' trajectories. After tracks were constructed, they were re-inspected manually prior to storing the raw coordinates for further data processing. A migration class package was written with Python software and the net distance (distance between starting point and end point) and persistence (net distance divided by the overall distance) of the movement were calculated.

Tie2 ± CD34 ± Integrin staining protocol Melanoma samples or specimens from the endothelial xenograft assay were deparaffinized and antigen retrieval was achieved with Proteinase K (Gerbu) incubation (20µg/ml in TE-Buffer; RT; 6min). Thereafter, specimens were incubated with Avidin and Biotin solutions (Dako Cytomation Biotin Blocking System; 10min; RT) and washed with TBST. Samples were incubated with mouse IgG blocking reagent for 1h at RT (MOM Kit; Vector Laboratories). Following washings with TBST, buffer equilibration with MOM diluent, samples were incubated with mouse anti-human CD34 at 4°C over night (1:50 in MOM diluent). TBST wash was followed by application of anti-mouse IgG reagent for 10min at RT. Next, the specimens were washed with TBST and incubated with Alexa488 coupled Streptavidin (Molecular Probes). For Tie-2 staining, the samples were additionally blocked with 10% normal goat serum (Invitrogen; ready-to-use; 1h; RT). Rabbit anti-mouse/human Tie-2 antibody was applied (1:100 in PBS with 3% normal goat serum; over night; 4°C). After TBST washing, goat-biotinylated anti-rabbit antibody was used (1h; RT). A brief TBST wash was followed by application of Alexa-546 coupled Streptavidin (Molecular Probes). In some experiments, integrins were stained in parallel. Integrin antibodies were conjugated with Alexa405 using a labeling kit (Zenon). The kit was used according to the manufacturer's instrucitons. Following washings, samples were mounted with cover slips using Fluoromount G and studied by confocal microscopy (Leica TCS SP5). Images are shown in pseudocolors.

p-FAK [Tyr397] staining in spheroids Spheroid assays were performed as described (3, 4). The spheroids were intensely washed with PBS (2 x for 1 h) at 4°C, followed by blocking of unspecific binding sites with 3% BSA, 10% donkey serum and 0.3% Triton-X. Following a brief wash, primary antibody (p-FAK [Tyr397] 1:200) was allowed to bind over night at 4°C. Again following a brief wash, the secondary antibody was allowed to bind over night at 4°C. Following intense washings (6 x a 20min) with TBST, the spheroids were transferred to confocal chambers and examined by confocal microscopy (Leica SP5).

Cornea pocket assay The cornea micropocket assay utilizes the normally avascular corneal tissue as a substrate for angiogenesis in response to specific and non-specific stimuli. Mice (8 week old C57BL/6J) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Using a 30-degree microknife, a partial-thickness incision in the mid-cornea was made. A von Graefe knife was inserted under the edge of the inferior lip of the partial-thickness incision to create the pocket and to lengthen it towards the inferior limbus. Equal size slow-release pellets were prepared by mixing appropriate amounts of growth-factor with 3 µl Hydron (12% (wt/wt) poly(2-hydroxyethyl methacrylate) / 99.5% ethanol) and 5 mg sucralfate and then spread on to a 4 x 4 mm grid containing 100 sub-sections. Pellets (0.4mm x 0.4mm) were implanted into the pocket at a distance of 1.0 ± 0.2 mm from the corneal limbus. The growth factor amounts per pellet were 80 ng of purified human (h)VEGF-A (293-VE; R&D Systems); 50 ng rAng-2 (R&D); 50 ng rAng-2 with a cocktail of integrin blocking antibodies, 50ng αvβ3-mAb, 50ng αvβ5-mAb, 50ng α5β1-mAb; 50 ng rAng-2 with 50ng IgG control antibody. The corneas were removed and stained 6 days after implantation of the pellets.

CD31 ± p-FAK[Tyr397] staining of cornea pocket tissue Corneas were fixed in 4% PFA overnight and washed with PBS+0,3%Triton for permeabilization. After blocking with goat serum, the primary antibodies rat anti mouse CD31 (BD Bioscience 553370/ 557355) and rabbit-anti-mouse p-FAK[Tyr397] (diluted 1:100) were added and incubated overnight at room temperature. Following washings with PBS and 0,3%Triton, secondary antibody staining was performed with goat anti rabbit Alexa 546, goat anti rat Alexa 488 and DAPI for nuclei.

Staining of 50µm sections of the xenografting assay The xenografting assay was performed as described (1, 2). Sections (50µm) were deparaffinized, followed by digestion of the Matrigel with a digestion solution (see digestion mix in the spheroid sprouting assay). After intense washings with TBST, antigen retrieval was achieved with Proteinase K for 10min at 37°C. Intense washings with TBST was followed by blocking for 1 h with 3% BSA, 10% donkey serum, 0.3% Triton in TBST. Both primary antibodies (CD31 and p-FAK[Tyr397] were allowed to bind overnight at 4°C). After washings, the corresponding secondary antibodies were incubated for 1 h at room temperature in combination with Hochest.

Quantification of the Matrigel assay The stained Matrigel plugs were scanned using a Zeiss SM-700 confocal microscope at a magnification of 20x with a resolution of 0.96µm/pixel. Stacks of the 50µm Matrigel plugs were recorded at a z-resolution of 0.5µm between slices. The z-stacks were processed using an open source ImageJ distribution

called the Fiji (http://fiji.sc/). In order to segment the images, the stacks were converted to image sequences and then processed using the “advanced weka segmentation” plug-in. Advanced weka segmentation is a machine learning algorithm that segments images based on a pre-selection of positive or negative classifiers. For this purpose, a database of 100 positive and negative classifiers was created in order to train the "weka" algorithm to recognize the vascular area in the subsequent images. The vascular area and density of the resulting binary images was then quantified using the “analyze particles” functionality of Fiji.
In order to analyze the network structure of the microvessels, the z-planes of the binary images were smoothened using the smooth (3D) plug-in (Gaussian blur radius of 1 pixel). The resulting images were then thresholded and skeletonized using the "skeleton(3D)" plug-in. Subsequently, the 3D skeletons were quantified by applying the "analyze skeleton(3D)" plug-in.

Retinal immunohistochemistry Postnatal mouse eyeballs were enucleated at the denoted time points, fixed with methanol, washed in PBS, and blocked in 0.5% Triton X-100 in BSA (1%) containing 10% goat serum for 1h at RT. Denoted primary antibodies were added and incubated over night at 4°C. After washings with PBS, appropriate secondary antibodies were incubated for 1h at RT. The retinas were studied by confocal microscopy (Leica TCS SP5 or Zeiss LSM700) to detect phosphorylated FAK, and in all other experiments with an Olympus XI81 microscope.

Retinal Ang-2 in situ hybridization and collagen IV immunohistochemical studies For whole-mount *in situ* hybridization (ISH) and immunohistochemistry (IHC), retinal cups were fixed in 4% paraformaldehyde (PFA)/PBS overnight on ice. After washing in 0.1% Triton-X/PBS (PBS-T), retinas were dehydrated and rehydrated through graded methanol series, and briefly digested with proteinase K followed by postfixation in 4% PFA/0.2% glutaraldehyde/PBS-T for 20 min at room temperature. The retinas were then hybridized with digoxigenin labeled Angpt2 cRNA probes (740-1764 of NM_007426) and were subsequently incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche). After the coloring reactions with nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (Roche), retinas were labeled with a polyclonal rabbit anti-type IV collagen antibody (COSMO BIO Co.) and Alexa Fluor 488-donkey anti-rabbit secondary antibody (Invitrogen). Images of ISH and IHC were taken separately in black and white with an Axioplan 2 microscope (Zeiss) equipped with associated software (AxioVision, version 3.1; Zeiss). For the merged images, the signals were pseudo-colored using Adobe Photoshop CS software (Adobe).

Spheroid assay for transcriptomic profiling HUVEC spheroids of defined cell number (400 cells) were generated as described (1, 2). The following day, 400 spheroids were embedded into 1ml of collagen or fibrin gel using 0.4U of thrombin for polymerization of fibrin gels. After polymerization, embedded spheroids were cultured without stimulation for 24h or they were stimulated with 25ng/ml VEGF-A or bFGF for 24h or 48h at 37°C, 5% CO_2, and 100% humidity.

RNA isolation and microarray processing Gels containing EC spheroids were directly homogenized in TRI reagent LS and total RNA was isolated according to manufacturer's instructions. The isolated RNA was further purified using RNeasy Total RNA Mini Kit (Qiagen, Hilden, Germany). Purified RNA of approximately 100 gels was pooled to obtain 15µg total RNA. An additional step of DNase digestion was used to avoid contamination with small amounts of genomic DNA. The quality of RNA samples was assessed using RNA 6000 Nano chips (Agilent, Santa Clara, CA) and quantiated by measuring the absorbance at 260nm. Preparation of cRNA was performed according to the protocols recommended by Affymetrix. Generally, 30 to 80µg of purified cRNA were obtained when using 8µg of total RNA. Then, cRNA samples were fragmented at 94°C for 35min (in 200mM Tris-Acetate, pH 8.1, 500mM potassium acetate, 150mM magnesium acetate). Three biological samples (2 x collagen, 1 x fibrin) were collected. The RNA of each biological sample was split and hybridized in duplicate. Aliquots of 10µg of each sample were hybridized to Affymetrix HG-U133A and B arrays and processed according to the Affymetrix protocol. Arrays were scanned on an Affymetrix GeneChip scanner.

Analysis of microarray chip data Array data were analysed with the GECKO software. Replicate hybridizations were normalized and merged for further ratio calculation. Element by element, ratios were computed from two experimental datasets creating a new dataset containing ratios and p-values. Genes were considered differentially expressed if ratios for qualifiers were regulated by a factor two with a p-value lower than 0.01. Qualifiers found to be not significantly different from background were manually removed. Hierarchical clustering was performed using Spotfire software based on the UPGMA (unweighted average) clustering method.

qRT-PCR Total RNA (1µg) was transcribed into cDNA with the reverse transcriptase kit from Qiagen according to the manufacturer's protocol. The cDNA was diluted 1:50 and 5µl were used for each PCR reaction. Primer (stock 100pmol/µl; dilution 1:50; TEK forward: CCAGGATGGCAGGGGCTCCA, reverse: GGTAGCGGCCAGCCAGAAGC; Ang-2: forward: TTCAGTTCTTCAGAAGCAGCAACA, reverse: AGCAACAGTGGGGTCCTTAGCTG; ITGA1:

forward: TGGTCCCGAGATGTGGCCGT, reverse: GGGTGACACGGTACTGCAAATCAGC; ITGA3 forward: GGCCCACCTGGTGTGACTTCTT, reverse: TTGGTCCCGAGTCTGCCAGC; ITGA5: forward: CGGATAGAGGACAAGGCTCA, reverse: CCACCCTCACCCACATTC; ITGAV: forward: GCTTTTATTGGCAAGGTCAG, reverse: CAGCCACAGAATAACAA; ITGB1 forward: CGTAGCAAAGGAACAGCAGAGAGAAG, reverse: TTGAGTAAGACAGGTCCA-TAAGGTAGTAG; ITGB3 forward: CCTCATCCATAGCACCTCCACATAC, reverse: CCATCATTCCATCCTGGCACTTATTC; ITGB5 forward: AAATGTGCCTGGTGCTCCAAAG, reverse: AATCTCCTGTGGTGTCATCTGAATG) and the qPCR Mix (Thermo Fisher) were added and the samples were analysed with the 7300 Real-Time PCR System from Applied Biosystems. Annealing temperature was 60°C in all cases. HMBS, h18S, GAPDH, PDH, actin or HMBS were used as housekeeping genes.

References

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