Supplementary material

Supplementary methods

sR3 ELISA

SR3 protein expression levels were analyzed in the serum of untreated and carcinogen treated transgenic mice using enzyme-linked immunosorbent (ELISA) assays, using wild-type mouse serum as a negative control. Plates (96-well, Nunc Maxisorp) were coated with 0.5 µg/ml anti-human IgG capture antibody (Sigma) in 0.1 M $NaHCO_3$, pH 9.5, washed using Tris-buffered saline with 0.05% Tween (TBST) and blocked with 1% bovine serum albumin (BSA, Sigma). SR3 was allowed to bind to the antibody coated and blocked well surface. Bound sR3 was detected using goat anti-human VEGFR-3 antiserum (R&D Systems), followed by horseradish peroxidase-conjugated streptavidin and tetramethylbenzidine (TMB) substrate. After addition of H_2SO_4 to 1.3 M, absorbance was measured at 450 nm. Average absorbance values of wells not coated with anti-human antibodies and of wells without anti-human VEGFR-3 antibodies were similar to wells incubated with the buffers, TMB and H_2SO_4 only. The latter values were subtracted from the average values of sample wells to correct for background absorbance.

Immunofluorescence staining of tissue sections

Primary antibodies against the following mouse epitopes were used: CD11b (Invitrogen), MECA32 (BD Bioscience), CD45 (R&D Systems), F4/80 (Serotec), CD4 (BD Bioscience), CD8 (BD Bioscience), VEGFR-3 (R&D

Systems), CD68 (Abcam), Nrp-1 (R&D Systems) and Nrp-2 (Millipore). Images obtained using an Axioscope2 mot plus microscope with an AxioCam MRc camera (Zeiss) were analyzed using ImageJ software. The number of CD11b, CD68, CD4 and CD8 stained cells was counted per dermal area. F4/80 stained cells were quantified as percentage of dermal area stained using the F4/80 antibody, due to the high density of these cells.

Fluorescence-activated cell sorting (FACS)

Samples of 3 cm x 3 cm of back skin were digested using collagenase IV (Gibco) in 1mM $CaCl_2$, PBS at 37°C. The spleen of a wild-type mouse was used for setting fluorophore color compensations. For macrophage sorting, instead of the spleen, CompBeads (BD Pharmingen) were used as compensation controls. All samples were incubated with an anti-CD16/32 antibody (Biolegend), to block unspecific antibody binding to FcγR III/II. FITC-, PE-, PerCP- or APC-conjugated antibodies raised against the following mouse epitopes were used: CD45 (BD Bioscience), CD11b (BD Bioscience), F4/80 (eBioscience), Ly-6C/G (BD Bioscience), Tie2 (eBioscience), CD206 (AbD Serotec), CD4 (BD Bioscience), CD8 (Biolegend). Isotype control antibodies (BD Bioscience) were included for each antibody and each genotype in each condition analyzed. Fluorophore color compensations were calculated using the FACS Diva software (BD Biosciences). For analysis of macrophage populations, flow was recorded for 60 seconds at constant speed using a BD FACSCanto device with BD FACS Diva software. An FSC-SSC gate was set on the cells and the numbers of stained cells were counted as percentage of all gated events. Cell populations were analyzed using FlowJo

software. For the collection of macrophages ($CD45^+$, $CD11b^+$, $F4/80^+$ cells) for gene expression analysis, all cells in the samples were sorted using the BD FACSAria IIu cell sorter.

Analysis of $CD4^+$ and $CD8^+$ T cells was performed after migration of the cells out of back skin samples. 3 cm x 3 cm back skin samples were collected from mice treated once with DMBA and 14 times weekly with TPA. Subcutaneous adipose tissue was carefully removed using a scapel blade. The samples were incubated overnight in RPMI-Glutamax (Gibco), 10 % FBS (Gibco), 1% antibiotic anti-mycotic (Invitrogen) at 37°C. Cells that had migrated into the medium were collected, stained and analyzed using flow cytometry as described.

To analyze blood leukocyte and monocyte counts, blood was withdrawn by cardiac puncture and quickly transferred to EDTA tubes (Mini Collect, Greiner-Bio One). 470 µl of EDTA anticoagulated blood was transferred into 10 ml ice-cold erythrocyte lysis buffer (1.54 M NH_4Cl, 100 mM $KHCO_3$, 1mM EDTA, pH 7.3). 100 µl AccuCheck counting beads (Invitrogen) were added to all samples to normalize for loss of sample volume during processing and cytometer flow rate in the analysis. Anti-CD16/32 antibody was used to block binding to FcγR III/II. Blood leukocytes and monocytes were stained with antibodies against mouse CD45, CD11b and Ly-6C/G (all from BD Bioscience). Samples were analyzed using a BD FACSCanto cell sorter. CompBeads (BD Pharmingen) were incubated with antibodies and used as compensation controls. Gating of positive cell populations was set based on

isotype control. Relative leukocyte and monocyte counts were obtained by normalizing the number of $CD45^+$ cells or $CD11b^+$,Ly-6C/G^+ cells, respectively, to the number of counting beads in the analyzed sample volume.

RNA isolation, reverse transcription, product amplification and purification

RNA was isolated from freshly FACS-sorted $CD45^+$, $CD11b^+$, F4/80^+ cells using the RNeasy Mini Kit (QIAGEN), according to the manufacturer's recommendations. The cDNA was synthetized from RNA isolated from skin samples using the High Capacity Reverse Transcription Kit (Applied Biosystems). RNA isolated from FACS-sorted $CD45^+$, $CD11b^+$, F4/80^+ cells was reverse transcribed and the obtained cDNA was amplified using the Ovation Pico WTA System (NuGEN, CA) according to the manufacturer's recommendations. Amplified cDNA was purified using the DNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's recommendations.

Real-time polymerase chain reaction

Taqman-Probe (Applied Biosystems) or SYBR Green (Sigma-Aldrich) technology-based assays were used with FastStart Universal Probe Master or FastStart Universal SYBR Green Master (Roche) reagents, respectively, in a 7900 HT Fast Real time PCR System (Applied Biosystems). Triplicate reactions were set up using ribosomal protein 36B4 (RPLP0) as an internal control gene. Fold change values were calculated by the $\Delta\Delta C_T$-method setting the fold change of the internal control gene as 1 and the derived

average ct-value of the wild-type mouse group as 1 in comparisons between study groups.

Primary keratinocyte culture and proliferation assay

Wild-type FVB mice were used to obtain primary keratinocytes for *in vitro* proliferation assays. The mice were sacrificed, shaved and washed in iodine (Betadine, SwissMedic) and in 70 % ethanol. The body skin was removed, subcutaneous fat scraped off and the skin floated in an antibiotic anti-mycotic solution (Gibco) for 5 min. Thereafter, the skin was floated in 0.8 % trypsin, PBS. The epidermis was scraped off and transferred into DNase medium (Sigma-Aldrich) and incubated under agitation at 37°C for 30 min. Subsequently, the solution was filtered through a 70 µm cell strainer and centrifuged. The cell pellet was washed with DNase medium and finally suspended in culture medium (MEM, 5 µg/ml insulin, 10 ng/ml EGF, 10 µg/ml transferrin, 10 µM phosphoethanolamine, 10 µM ethanolamine; all from Sigma-Aldrich; 0.36 µg/ml hydrocortisone, Calbiochem; glutamine and penicillin/streptomycin, Invitrogen; chelated FCS, Gibco). Cells were counted in a Neubauer chamber and 10^6 cells were plated per well into fibronectin-collagen (Invitrogen, Advance BioMatrix) coated wells of a 96 well plate at 34°C, 5 % CO_2. After 16 hours, cells were cultured further in EGF-free culture medium. Growth factors were added as follows: epidermal growth factor (59 ng/ml, Sigma), 200 ng/ml human VEGF-C (a kind gift from Dr. Michael Jeltsch, University of Helsinki, Finland), 200 ng/ml mouse VEGF-D (R&D Systems) or 1.5 µg/ml bovine serum albumin (Sigma). After 48 hours of culture with the analyzed factors, cells were incubated with 10 µg/ml 4-

methylumbelliferyl-heptanoate (MUH) and fluorescence (355 nm excitation, 460 nm emission) was read 1 hour later. The fluorescence measured is directly correlated to the number of viable cells (31). Triplicate wells were used for each factor; the values were averaged and background corrected by subtraction of fluorescence value of the medium and MUH. One-way ANOVA with Tukey post-hoc test was used for statistical analysis of the results.

The activity of VEGF-C and VEGF-D was tested in a similar manner using lymphatic endothelial cells obtained from 'immorto-mouse' (32) skin cultured at 37°C in 96-well black clear-bottom plates (Costar) coated with 10 µg/ml fibronectin (BD Biosciences) and collagen (Inamed, Fremont, CA), in DMEM/F-12 medium (Invitrogen) supplemented with 20% FBS, heparin (Sigma), and EC mitogen (AbD Serotec).

Production of skin and tumor homogenates for cytokine analyses

The back skin and papilloma samples were homogenized in Complete Mini EDTA free (Roche), 0.6 M NaCl, 0.2 % Triton X-100, 0.5 % BSA, Tris-HCl (pH 7.4) for 2 min and sonicated at 25 Hz. Protein concentrations in supernatants were measured using the Pierce BCA kit (Thermo Scientific) according to the manufacturer's recommendations. Cytokine levels in homogenates of papillomas collected from wild-type and transgenic mice after long-term carcinogenesis were compared semi-quantitatively using a mouse cytokine array kit (R&D Systems) according to the manufacturer's recommendations. Based on this array, potentially differentially expressed cytokines (MCP-1/CCL2, G-CSF, MIP-1α/CCL3, MIP-2, TNF-α, M-CSF, IL-16, TREM-1) were quantitated using a multiplexed particle-based flow cytometric cytokine assay

(33). Additionally, the levels of selected macrophage polarization related cytokines (VEGF-A, IL-12/p40, IL-4, IL-6, IL-10) were analyzed in the back skin samples of wild-type and transgenic mice treated with DMBA followed by 14 weekly TPA treatments. Cytokine kits were purchased from BioRad (Ismaning) and R&D Systems (Oxon). The analysis was conducted using a conventional flow cytometer (Guava EasyCyte Plus, Millipore). TREM-1 was analyzed with an ELISA Kit (R&D Systems). TNF-α levels were measured in back skin samples of mice treated once with DMBA and 14 times weekly with TPA (tissue collection 7 hours after last TPA treatment), using an ELISA with two anti-TNF-α antibodies (Invitrogen and biotinylated from eBioscience), streptavidin-horseradish peroxidase (Amersham Bioscience).

Measurement of blood vascular leakage

Vascular leakage was quantified as described previously in ears treated with TPA (3 weekly treatments), untreated ears and treated back skin after one DMBA treatment followed by 3 or 14 weekly TPA treatments. Imaging was done 24-30 hours after the last TPA treatment. The mice were maintained under isoflurane anesthesia on a heated platform in an IVIS Spectrum imaging system (Caliper Life Sciences) during the imaging. A sequence of 3 s exposure images (excitation 745 nm, emission 800 nm, binning of 4) was initiated and paused after the first image was acquired. 100 μL per 20 g mouse weight of 10 μmol/L P20-800 tracer (polyethylene glycol coupled to the near-infrared dye IRDye 800 CW, a kind gift from Dr. Paola Luciani, ETH Zurich) solution was injected intravenously into the tail vein and the imaging sequence was immediately resumed. Images were acquired every 15 s for a

total imaging time of 20 min. Regions of interest (ROIs) over each ear, treated back skin area and the popliteal vein were drawn and signal intensity values at each time point in fluorescent counts were determined using Living Image 4.0 software (Caliper). Leakage rates were quantified by normalizing the signal intensity values of the ear or back region of interest (ROI) to the value of the popliteal vein ROI for each time point to adjust for injected dose and the dynamics of intravascular signal. Tissue leakage rates (expressed in min^{-1}) were defined as the slope of the ear or back ROI intensity divided by the popliteal vein ROI intensity plotted over time.

Supplementary figure and table legends

Suppl. Table 1. Levels of macrophage- and macrophage polarization-associated cytokines. Values are pg of cytokine per mg of total protein, N=5. MCP-1: monocyte chemotactic protein protein 1; CSF: colony stimulating factor; G-CSF: granulocyte-CSF; MIP: macrophage inflammatory protein; M-CSF: macrophage-CSF; TREM-1: triggering receptor expressed on myeloid cells 1.

Suppl. Fig. 1. SVEGFR-3 in serum, tumor growth and malignant transformation and VEGFR-3 ligand effects on epidermal cells. a) Concentration of the sVEGFR-3 protein in mouse sera in the basal state and after 21 weeks of carcinogenesis treatment (N=2). b) Tumor growth rate (ratio of small papillomas to large papillomas) in wild-type and sVEGFR-3 transgenic mice (N=28). c) Malignant conversion rate (number of SCC divided

by number of large papillomas) in wild-type and sVEGFR-3 transgenic mice (N=28). WT = wild-type, TG = transgenic, **<0.01. d) Effect of growth factors on the proliferation of mouse epidermal cells. Mouse primary epidermal cells were cultured in basal medium or medium supplemented with epidermal growth factor (EGF, 59 ng/ml), VEGF-C (200 ng/ml), VEGF-D (200 ng/ml) or bovine serum albumin (BSA, 1.5 ug/ml). After culture with the factors (48 hours), cell numbers were compared by the incorporation of MUH ("Absorbance"), as detailed in the supplementary materials and methods. N≥7, *<0.05

Suppl. Fig. 2. Blood leukocyte and monocyte counts. Blood leukocytes and monocytes were quantified by flow cytometry, after normalizing cell counts to blood volume by using counting beads.

Suppl. Fig. 3. Immunofluorescence staining of $CD45^+$,$CD4^+$ and $CD45^+$,$CD8^+$ T cells in DMBA-TPA treated skin of wild-type, control transgenic and transgenic mice. Note unspecific green fluorescence in the stratum corneum of the epidermis.

Suppl. Fig. 4. Macrophage polarization. a) Gating of $Tie2^+$, $CD206^+$ cells from $CD45^+$, $CD11b^+$ cells obtained from DMBA-TPA treated skin of the wild-type and transgenic mice. b) Mean fluorescence intensity plots for Tie2 and CD206 expression. Macrophages obtained from transgenic skin express less Tie2 than macrophages from wild-type skin. c) Expression of macrophage

polarization associated genes in $CD45^+$, $CD11b^+$, $F4/80^+$ cells isolated by fluorescence assisted cell sorting from carcinogenesis treated skin.

Suppl. Fig. 5. VEGR-3, MECA32 and CD68 expression in DMBA-TPA treated skin and tumors. a) Back skin DMBA-TPA treated for 14 weeks and SCC stained using blood vascular marker MECA32 (red) and VEGFR-3 (green) antibodies. Note that VEGFR-3 is expressed on blood vessels in chronic inflammation and in few peritumoral blood vessels. b) SCC stained using CD68 (red) and VEGFR-3 (green) antibodies. Note the absence of VEGFR-3 expression in the $CD68^+$ cells. Scale bars 100 µm.

Suppl. Fig. 6. Expression of neuropilins in skin and tumors of wild-type mice. a) Nrp-2 (red) and CD68 (green) staining in untreated skin and SCC skin and papilloma. Note that the expression of CD68 and Nrp-2 do not overlap. b) Nrp-1 (green) and CD68 (red) expression in skin after 14 weeks of DMBA/TPA treatment. Note that the expression of CD68 and Nrp-1 do not overlap. c) Nrp-1 (green) and MECA32 (red) expression in untreated skin, skin after 14 weeks of DMBA/TPA treatment, and papilloma. Scale bars 100 µm.