

Tumor-type-dependent effects on the angiogenic abilities of endothelial cells in an *in vitro* rat cell model

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Abstract. Adequate vascularization is pivotal for tumor progression and metastasis. Tumor angiogenesis is based on a sequence of interactions between the tumor and surrounding cells and the extracellular matrix. It is widely known that a tumor can influence and control its surroundings to create favorable conditions for further growth. To investigate the influence of various tumor types on endothelial cells (ECs), an *in vitro* rat cell model was used and rat liver EC52 cells were co-cultured with conditioned medium derived from breast cancer MCR86, osteosarcoma ROS-1, colon cancer CC531 and rhabdomyosarcoma R1H cell lines. In a distinct tumor-type-dependent manner, the EC52 cells exhibited changes in their function and gene expression. In

all functional cell culture assays (proliferation, migration, transmigration, invasion and tube formation) the breast cancer cells exerted a significant effect on the angiogenic abilities of the ECs. When comparing the various tumor cell types, only the breast and colon cancer cells led to a significant stimulation of the EC migration and invasion. Proliferation, migration, invasion and tube formation were not or only hardly influenced by the osteosarcoma or rhabdomyosarcoma cells. Similarly, the breast and colon cancer cells exhibited the strongest influence on the upregulation of EC angiogenic genes, including the ones encoding vascular endothelial growth factor A, platelet and endothelial cell adhesion molecule 1, fibroblast growth factor 2, Von Willebrand factor, C-X-C motif chemokine ligand 12 and tyrosine kinase with immunoglobulin-like and EGF-like domains 1. Therefore, it is hypothesized that tumor cells enhance the angiogenic properties of ECs, including proliferation, migration, invasion and tube formation in a tumor-type-dependent manner. This is likely based on the upregulation of pro-angiogenic genes in ECs induced by varying cytokine secretion signatures of tumor cells.

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Abbreviations: EC, endothelial cell; FCS, fetal calf serum; CM, conditioned medium; PECAM-1, platelet endothelial cell adhesion molecule-1; VEGF, vascular endothelial growth factor; FGF2, fibroblast growth factor 2; VWF, Von Willebrand factor; CXCL12, C-X-C motif chemokine ligand 12; TIE1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; PDGFRβ, platelet-derived growth factor receptor β; EPC, endothelial progenitor cell; MCP-1, monocyte chemo-attractant protein-1; MIP-2, macrophage inflammatory protein 2-α; TRAIL, TNF-related apoptosis-inducing ligand

Key words: tumor vascularization, endothelial cells, tumor microenvironment, colon cancer, breast cancer, osteosarcoma, rhabdomyosarcoma, pro-angiogenic factors

Introduction

Tumors develop as organ-like structures with a high metabolic demand (1). In the early stages, their growth depends on the passive diffusion of oxygen, nutrients and metabolic waste (2). When a tumor reaches 1-2 mm in diameter and its demand for oxygen and nutrients surpasses the local supply, hypoxia and nutrient starvation trigger an 'angiogenic switch' to allow for tumor progression (3). The further enlargement of these growths depends on angiogenesis initiated by cancerous cells to enhance the supply of oxygen and nutrients (4). Tumor angiogenesis involves a complex series of events, starting with vessel membrane degradation, followed by endothelial cell (EC) migration, proliferation and re-organization, promoting vessel maturation, indicating that ECs are essential in each step of angiogenesis (5).

As ECs migrate towards a tumor, they can form loops and branches to connect with it and supply it with nutrients (6). This mechanism involves a complex interaction between many factors, including adhesion proteins, growth factors, junctional molecules, oxygen sensors and endogenous inhibitors (7). The crosstalk between tumor cells and ECs leads to modification of their properties and facilitation of the tumor cell function (8). It has been demonstrated that the tumor microenvironment contains a variety of cytokines regulating its angiogenesis (3). Pro- and anti-angiogenic molecules, necessary for blood vessel growth, are not only produced by tumor cells themselves, but can also be products of ECs, pericytes, the extracellular matrix and plasma clotting (9,10). This complex interplay between stimulatory and inhibitory angiogenic factors is most likely influenced by the tumor cells in order to initiate and further support the vascular growth within the tumor (3). However, the underlying mechanisms and any tumor type variations remain unclear.

In the present study, the influence of various tumor types was analyzed, including that of breast cancer MCR86, osteosarcoma ROS-1, colon cancer CC531 and rhabdomyosarcoma R1H cells on EC52 cells in an *in vitro* rat cell model. Important steps occurring during tumor vascularization were examined, including EC proliferation, migration and tube formation, as well as subsequent underlying changes in genetic expression patterns. Investigation of tumor-induced angiogenesis may provide an improved understanding of tumor vascularization and progression, which in turn may lead to the development of novel anti-angiogenic therapeutic strategies in the future.

Materials and methods

Cell lines and cell culture. Rat colon adenocarcinoma CC531 cells (CLS Cell Lines Service GmbH, Eppelheim, Germany) were cultured in RPMI-1640 medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS; Biochrom Ltd., Cambridge, UK) and 2 mM L-glutamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Rat rhabdomyosarcoma R1H cells were obtained from Dr A. Raabe (Laboratory for Radiobiology and Experimental Radio-Oncology, University Cancer Center Hamburg, Hamburg, Germany) and were cultured in Dulbecco's modified Eagle's medium (Biochrom Ltd.) supplemented with 5% FCS. Rat mammary carcinoma MCR86 cells were provided by Professor P. J. K. Kuppen (Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands) and were cultured in RPMI-1640 medium supplemented with 10% FCS. Rat osteosarcoma ROS-1 cells were provided by Professor T. L. M. ten Hagen (Laboratory of Experimental Surgical Oncology, Department of Surgery, Erasmus MC, Rotterdam, The Netherlands) and were cultured in minimum essential medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% FCS. Rat liver EC52 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 1 μ M dexamethasone (PeloBiotech GmbH, Planegg, Germany). All cell lines were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed every 2-3 days and the cells were passaged when 80-90% confluence was reached.

Direct co-culture of EC52 and tumor cells. A total of 2×10^4 tumor cells, including colon cancer CC531, rhabdomyosarcoma R1H, breast cancer MCR86 and osteosarcoma ROS-1 cells, were labeled with 25 μ M CellTrace™ Oregon Green® 488 (Thermo Fisher Scientific Inc.), and 2×10^4 EC52 cells were labeled with 2 μ M PKH26 (Sigma-Aldrich; Merck KGaA), according to the manufacturers' protocols. Equal numbers of tumor cells and EC52 cells were mixed and seeded in 6-well plates. The co-cultured cells were incubated in a 1:1 mixture of tumor cell and EC52 cell culture media. Images were captured after 3-4 days using a fluorescent Olympus IX83 microscope and were analyzed with CellSens Dimensions 1.16 software (both from Olympus Corp., Tokyo, Japan).

Preparation of tumor-conditioned medium (CM). For tumor-CM, tumor cells were grown in their normal cell culture medium in cell culture flasks with an area of 75 cm². When the cells reached ~80% confluence, the medium was changed and the cells were cultured for another 48 h. The supernatant was collected and filtered (pore size, 0.22 μ m). The CM was mixed with fresh EC culture medium in a 1:1 ratio and used for the cell culture assays (termed tumor-CM). A 1:1 mixture of non-conditioned tumor cell and EC culture media was used as a negative control.

WST-8 cell proliferation and viability assay. To assess the effect of tumor-CM on EC52 proliferation, the quantitative colorimetric WST-8 assay (PromoCell GmbH, Heidelberg, Germany) was performed at 3 time-points. Briefly, EC52 cells were seeded at a density of 1×10^3 cells/well in 100 μ l EC culture medium in 96-well culture plates in triplicate. After 24 h of incubation, the medium was replaced with tumor-CM or control medium. After 24, 36 and 48 h of incubation, 10 μ l WST-8 tetrazolium salt was added to each well for 2 h at 37°C. For each measurement time-point one culture well plate was prepared. The absorption was measured at 450 nm with a reference wavelength of 600 nm. After measurement, the culture well plate was discarded. The data was analyzed using SkanIt™ software version 3.2 (Thermo Fisher Scientific Inc.). The assay was performed 3 times.

Transwell migration and invasion assays. Transmigration and invasion assays were performed in a 24-well Transwell system (BD Biosciences, Franklin Lakes, NJ, USA) with 8- μ m-pore polycarbonate membrane inserts. Transmigration was performed 2 times in technical duplicates. For statistical analyses the single technical duplicates were used. For the invasion experiments, the Transwell chambers were coated with 40 μ l growth factor reduced Matrigel® (Growth Factor Reduced Basement Membrane Matrix; Corning Inc., Corning, NY, USA) and incubated for 60 min at 37°C. Invasion was performed 3 times. A total of 1×10^5 EC52 cells/well were seeded into the upper chambers in 200 μ l EC culture medium and 700 μ l tumor-CM or control medium was added to the bottom chambers. Following incubation at 37°C for 8 h, the cells remaining on the surface of the membrane were wiped away using a cotton swab. The cells that migrated through the membrane were fixed with ice-cold methanol and stained with 1 μ g/ml DAPI (Roche Molecular Diagnostics, Pleasanton, CA, USA). The stained cells were scanned and quantified

in 4 random fields per membrane (1 image per quadrant) at an x40 magnification using an Olympus IX83 microscope. In a few wells only 3 regions of interest (ROIs) could be evaluated due to technical problems such as unclear images.

Wound-healing assay. A wound-healing assay was performed in 12-well plates. EC52 cells were seeded at a density of 1×10^5 cells/well in EC culture medium. When the cells reached confluence, a scratch was created using a 100- μ l pipette tip. The cell layer was washed with PBS and CM or control medium was added. Images were captured at 0, 6, 12 and 24 h. The uncovered area of the scratch was measured in ≤ 4 ROIs in each well and compared with the measurement at 0 h, which was set to 1. In certain wells, not all ROIs could be evaluated due to unequal cell growth at the borders of the well. The assay was performed 3 times.

Tube formation assay. Matrigel® (10 μ l) was applied to each well of a μ -slide (a chambered coverslip) (ibidi GmbH, Martinsried, Germany) and incubated at 37°C for 30 min for gel polymerization. EC52 cells were seeded in technical triplicates at a density of 1.5×10^4 cells/well in 50 μ l tumor-CM or control medium on the surface of the gelled matrix. After 7 h, the cells were stained with Calcein-AM (Sigma-Aldrich; Merck KGaA) and images were captured with an Olympus IX83 microscope and processed using CellSens software. Capillary-like tube formation was analyzed using the WimTube analysis software (Wimasis GmbH, Munich, Germany) regarding covered area, tube length, branching points and loops. Tube formation was performed 3 times.

To detect the direct interaction of tumor cells and EC52 cells, 3.9×10^3 CM DiI dye-labeled tumor cells (staining solution 5 μ g/ml; Thermo Fisher Scientific Inc.) and 1.3×10^4 Oregon Green-labeled EC52 cells were seeded in direct co-cultures in Matrigel®-coated wells, as described above, and incubated for 6 h in a 1:1 mixture of tumor-CM and EC52 cell culture medium. A monoculture of EC52 cells was grown as the control. The evaluation was performed as described above. Direct tube formation was performed once with technical triplicates that were used for statistical analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the EC52 cells incubated in tumor-CM for 4 days using the RNeasy Mini kit with the corresponding QIAshredder Homogenizer (both from Qiagen GmbH, Hilden, Germany). The RNA was converted to cDNA using the QuantiTect Reverse Transcription kit with a DNase I incubation (15 min 42°C, 3 min 95°C) (Qiagen GmbH). The qPCR reactions were performed using the SsoAdvanced Universal SYBR-Green SuperMix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a Bio-Rad CFX96 Touch™ Light Cycler (Bio-Rad Laboratories, Inc.) and the following thermal cycling protocol: Polymerase activation and DNA denaturation: 30 sec 95°C, amplification: 40 cycles with denaturation at 95°C for 5 sec and annealing at 60°C for 30 sec, melt-curve analysis 55-95°C 0.5°C increment 5 sec/step. All kits were used according to the manufacturers' protocols and glucuronidase- β was used as the housekeeping control. The primer sequences used are listed in Table I. All reactions were performed in triplicate. The experiment was performed

3 times. The expression of individual genes was calculated and normalized by the $2^{-\Delta\Delta C_q}$ method (11).

Statistical analysis. The data are expressed as the mean \pm standard deviation. Statistical analysis was performed using SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Due to the small sample size with mostly $n=3$, non-parametric statistical tests were used since testing of normal distribution was considered to be negligible. In case of multiple comparisons, the Friedman test was used for dependent samples and the Kruskal-Wallis test was used for comparing independent samples. Mean differences between 2 independent samples were analyzed by the non-parametric Mann-Whitney U test; the asymptotic significance was used. Values of $P \leq 0.05$ were considered to indicate statistically significant differences.

Results

Effects of tumor-CM on EC52 morphology and proliferation. EC52 cells in direct co-culture with various tumor cells grew in colonies with uniform and characteristic cobblestone appearance. No morphological changes were observed in the ECs. The co-cultured cells grew evenly with each other with no signs of overgrowth or inhibition of either one of the cell populations. Where direct cell-cell contact occurred, different interaction patterns were observed. In the rhabdomyosarcoma R1H co-culture, double-labeled cells were visible, indicating close cell-cell interactions. In many areas of the colon cancer CC531 co-culture, the ECs formed a border around the tumor cell colonies. In the culture with the breast cancer MCR86 cells, the tumor cells began to grow in close contact with the ECs and grew around EC colonies in certain areas (Fig. 1A).

The effect of tumor-CM on the viability and proliferation of EC52 cells was investigated using a WST-8 assay at 24, 36 and 48 h of culture. The ECs proliferated over time in the tumor-CM as well as the control medium, mostly with a tendency of higher proliferation being observed in the tumor-CM (Fig. 1B-E). A significant increase in absorbance was observed when the ECs were cultured in R1H CM compared to control for 36 h ($P \leq 0.05$) (Fig. 1C), as well as in MCR86 CM compared to control at 36 h ($P \leq 0.05$; Fig. 1D).

Tumor-CM promotes the migratory capacity of ECs. To investigate the effect of tumor-CM on the migratory ability of ECs, 3 distinct cell culture assays were performed. First, a wound-healing assay was conducted. Compared with that of the control, the migration of EC52 cells into the scratched area was increased in the CC531 and MCR86 CM. This difference became significant in the MCR86 CM compared to control at time-point 12 h ($P \leq 0.05$; Fig. 2).

Subsequently, the influence of tumor-CM on the transmigration and invasion capacities of ECs was analyzed using Transwell assays. The transmigration was significantly stimulated in tumor-CM breast cancer MCR86 groups ($P \leq 0.05$; Fig. 3A and B). The invasion of the ECs through the Matrigel®-coated Boyden chambers was significantly stimulated by the colon cancer CC531 CM compared with the effect of the control ($P \leq 0.05$; Fig. 3C).

Table I. Primer sequences.

	Forward Primer (5'→3')	Reverse Primer (3'→5')
Tie1	AGGGTCCTGGAGACTGATCC	AAGGTACTGCATCCCGTTGG
Flt1	ACAGCAATGTGTTCCACAGCGT	TGGTTTCCTGCACCTGTTGCTT
Angpt2	AATGCACAGTAGCCCCCTTCC	GGTGCAGGCCTAAGTGATGT
Pecam1	GCAGACCCTTCCACGAAGAA	GCTTCGGAGACTGGTCACAA
Cxcl12	TCCGTGGGCTCTGAGTTTTC	GGAACCCAGAATCCCCACTG
Egf	TCGAGTCAACAAAGGGCCTC	GAGTACCAGATCTGCCGCTC
Egfr	CAACATCCTGGAGGGGGAAC	ATGTTTCATGGTCTGGGGCAG
Vwf	CCCGGGAAACTCCTTCTTCC	CAAGCAAGTCACTGTGTGGC
Pdgfrb	GCATTGGCTCCATTCTTCAT	CCGTGGTCATTACACTCAC
Fgf2	TCCATCAAGGGAGTGTGTGC	GGACTCCAGGCGTTCAAAGA
Vegfa	AATGATGAAGCCCTGGAGTG	ATGCTGCAGGAAGCTCATCT
Gusb	TGGCCTTGGCTTTGTGTACT	CGTGGGTGCTAGGAATCGAA

Stimulation of tube formation depends on direct cell-cell contact. To evaluate the angiogenic capabilities of ECs in a quantifiable manner, a tube-formation assay was performed. Following seeding on Matrigel®, the EC52 cells formed typical endothelial networks in the tumor-CM and control medium with no significant differences (Fig. 4A-D).

To further analyze whether the stimulation of tube formation depends on direct cell-cell interactions, ECs and tumor cells were seeded in direct co-culture on Matrigel®. By labeling tumor cells and ECs with different fluorescence dyes, the contribution of the different cell populations to tube formation could be visualized. The ECs directly co-cultured with breast cancer MCR86 cells displayed a significantly higher area, total tube length, number of branching points and total loops ($P \leq 0.05$) compared with the mono-cultured ECs (Fig. 4E-H). In addition, the total tube length was significantly longer in the rhabdomyosarcoma R1H group compared with that of the control ($P \leq 0.05$; Fig. 4F). Tumor cells were observed in close contact with the tubes formed by the ECs in all groups. Furthermore, the rhabdomyosarcoma R1H cells participated in forming tube-like structures by cell elongation and cell-cell interactions in close contact with the tubes formed by the ECs (Fig. 4I).

Tumor-CM upregulates angiogenesis-associated genes. Following incubation in tumor-CM for 4 days, the expression of 11 angiogenesis-associated genes in EC52 cells was determined by RT-qPCR (Fig. 5). In each group, the expression levels of angiogenic gene platelet endothelial cell adhesion molecule-1 (*PECAM1*) were significantly higher (except for ROS-1) in the tumor-CM compared with those in the control. The expression levels of other endothelial markers, including vascular endothelial growth factor A (*VEGFA*), fibroblast growth factor 2 (*FGF2*), Von Willebrand factor (*VWF*), C-X-C motif chemokine ligand 12 (*CXCL12*), tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (*TIE1*), VEGF receptor 1 (*VEGFR1*) and platelet-derived growth factor receptor β (*PDGFRB*) were significantly upregulated in groups stimulated with CC531 and MCR86 CM. *PECAM1* exhibited a ~2-6-fold higher expression level in the colon

cancer CC531 and breast cancer MCR86 groups compared with the rhabdomyosarcoma R1H and osteosarcoma ROS-1 groups. Whereas numerous angiogenesis-associated genes were upregulated in ECs that were cultured in the CC531 or MCR86 CM, only *PECAM1* and *CXCL12* in the R1H CM and *VEGFA* and *TIE1* in the ROS-1 CM were significantly upregulated compared with the control.

Discussion

Angiogenesis is necessary for tumor growth, development and metastasis (8). Tumor vascularization is a complex multistep process. Important components of this process are EC proliferation and migration, followed by the formation of mostly abnormal capillaries (12,13). ECs are stimulated by tumor-secreted factors to migrate and divide at the tumor sites, ultimately forming capillary structures stabilized by smooth muscle cells (14). Previous studies have demonstrated that. The tumor microenvironment serves a crucial role in its survival and development, and various factors influence its vascularization, however, the mechanism remains unclear (3,15). As part of the tumor microenvironment, direct interaction of cancer cells with ECs may be of great importance for tumor metastasis, but also for tumor angiogenesis (16). Considering the growing awareness of the role of the tumor microenvironment in its progression, the present study aimed at deciphering its effect on tumor vascularization by studying the changes in phenotype and gene expression. Furthermore, the functional properties of ECs under the influence of various tumor types were analyzed. The design of the present tumor/EC *in vitro* model was based on previous studies (17-19).

In the direct co-culture, signs of cell-cell interactions between the tumor cells and ECs were observed. It is widely known that ECs in tumors have a unique activated phenotype compared with those under normal conditions (3). In the colon and breast cancer groups, the cells began to surround each other, perhaps due to tumor cell-secreted soluble factors, such as tumor necrosis factor α , which can rearrange F-actin cytoskeleton filaments influencing the shape and motility of the cell (20), as well as transforming growth factor- β , which can

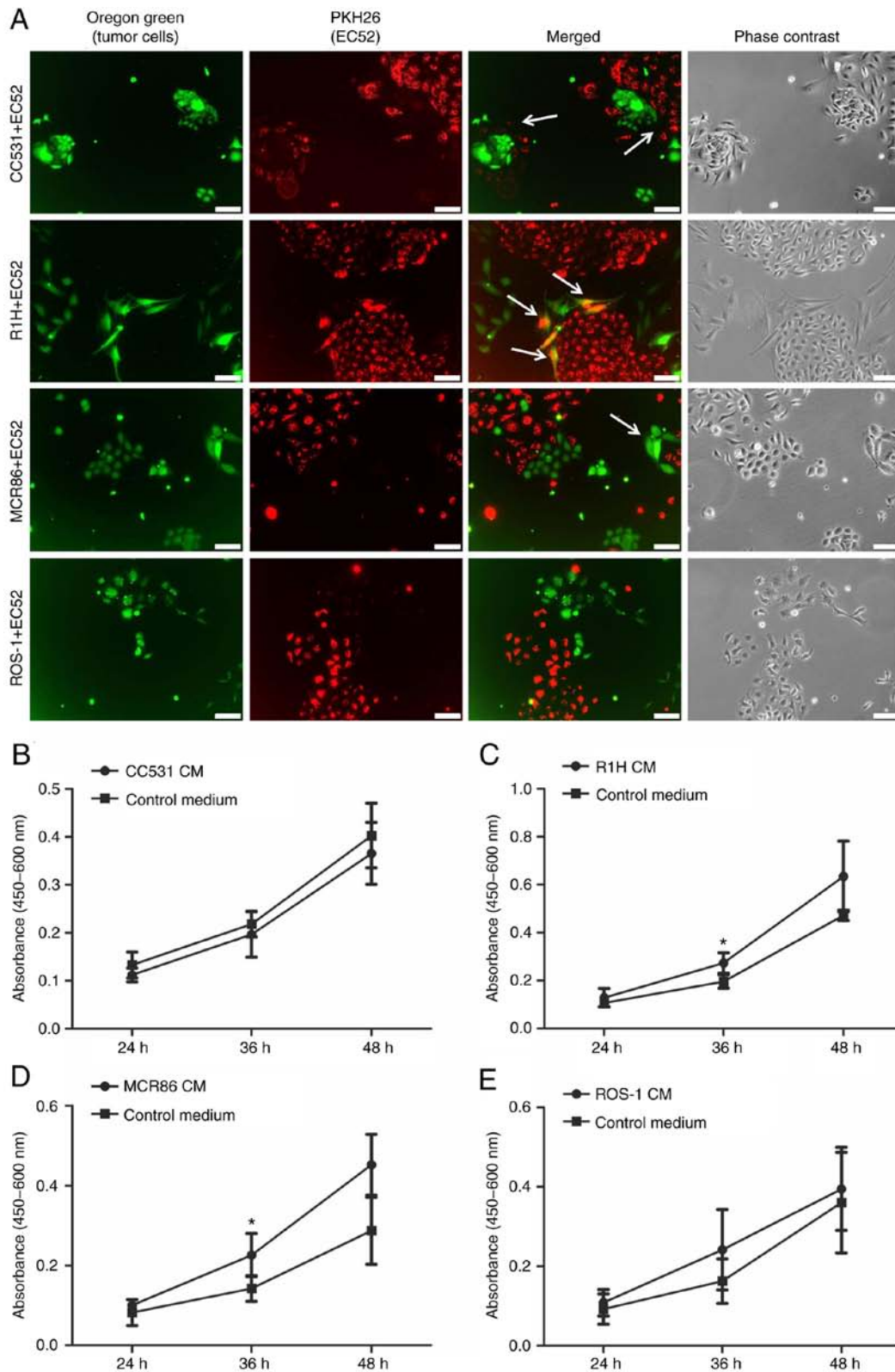


Figure 1. Morphology of EC52 cells in co-culture with tumor cells and their proliferation following culture in tumor-CM. (A) Representative images of EC52 cells co-cultured with tumor cells for 3-4 days. Oregon-Green-labeled tumor cells are indicated in green, and the PKH26-labeled EC52 cells are red. The arrows indicate direct cell-cell interactions and possible fusion can be observed. Scale bar, 100 μ m. (B-E) Quantification of EC52 cell proliferation following incubation in tumor-CM and control medium for 24, 36 and 48 h. Results are expressed as the mean \pm standard deviation. * $P \leq 0.05$. CM, conditioned medium.

alter the content of collagen type IV and provide a supporting structure for ECs (21,22). Although no distinct change in the morphology of the ECs could be detected, in certain areas they began to align with tumor cells in a manner similar to that

reported by a study on the interaction between umbilical vein ECs and human glioma cells (22). Similar findings revealed that the CM derived from breast cancer cells affected the phenotype and behavior of normal cells (21). After a number

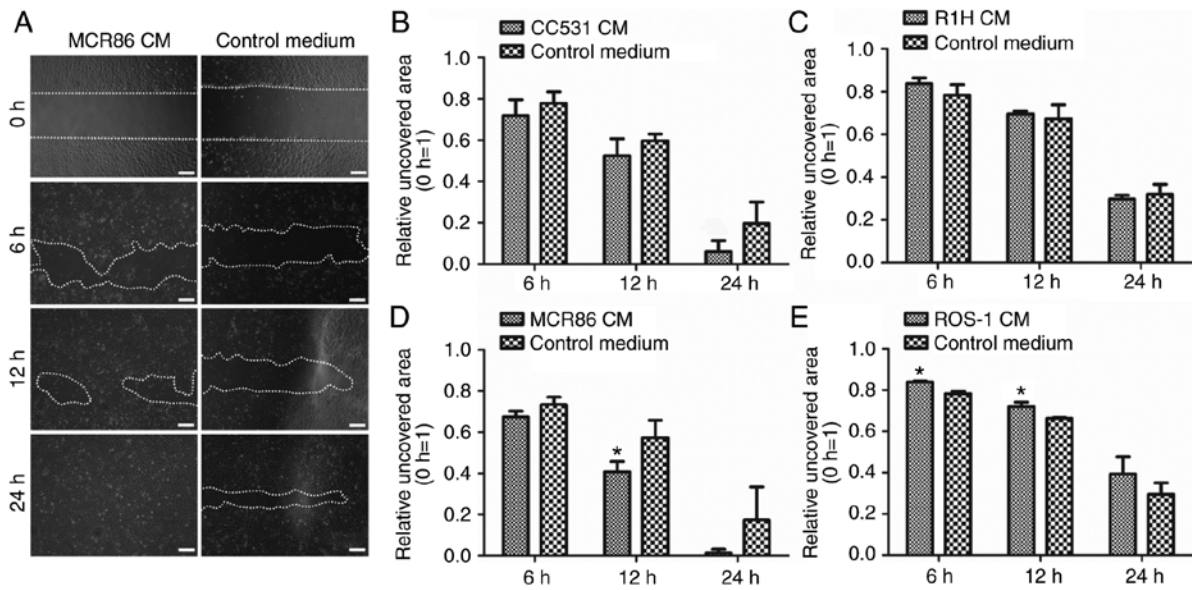


Figure 2. Effect of tumor-CM on the migration of EC52 cells. (A) Representative scratch test images of EC52 cell migration in breast cancer MCR86-CM and control medium. Scale bar, 200 μ m. (B-E) Quantification of the covered area of EC52 cells induced by tumor-CM and control medium after 6, 12 and 24 h. The bars represent the relative size of the uncovered area compared with that at time-point 0 h (data at 0 h is normalized to 1). The results are expressed as the mean \pm standard deviation. *P \leq 0.05. CM, conditioned medium.

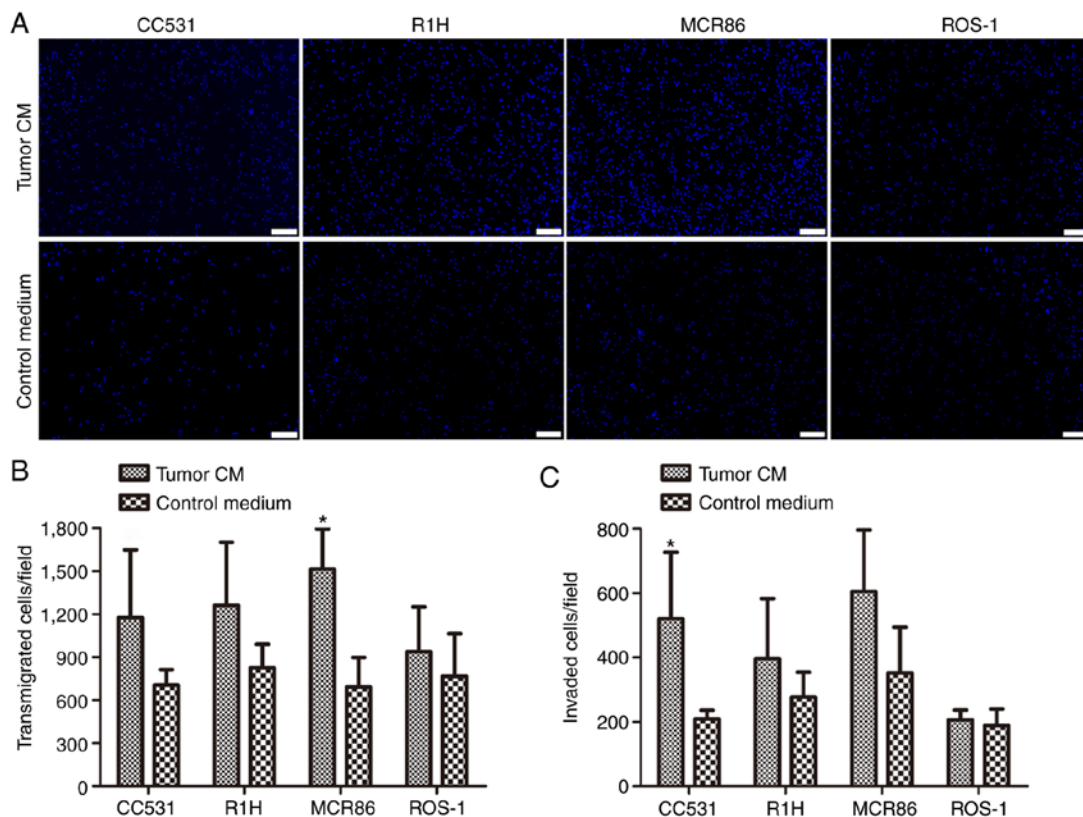


Figure 3. Effect of tumor-CM on invasion and transmigration of EC52 cells. (A) Representative images display the transmigration of EC52 cells stimulated by tumor-CM and control medium. Transmigrated EC52 cells are stained with DAPI (blue). Scale bar, 200 μ m. (B and C) Quantification of the transmigration and invasion of EC52 cells following stimulation with tumor-CM and control medium. The results are expressed as the mean \pm standard deviation. *P \leq 0.05. CM, conditioned medium.

of days of co-culture, double-labeled cells became visible in the rhabdomyosarcoma group. This could be an indication of cell fusion, although loss of dye or it being absorbed up by other cells cannot be excluded. Tumor cells have the ability

to fuse spontaneously with normal host cells, including ECs, which may alter the biological behavior of tumors (23). Cell fusion with macrophages or bone marrow-derived cells is quite a common feature of tumor cells to enhance metastasis, drug

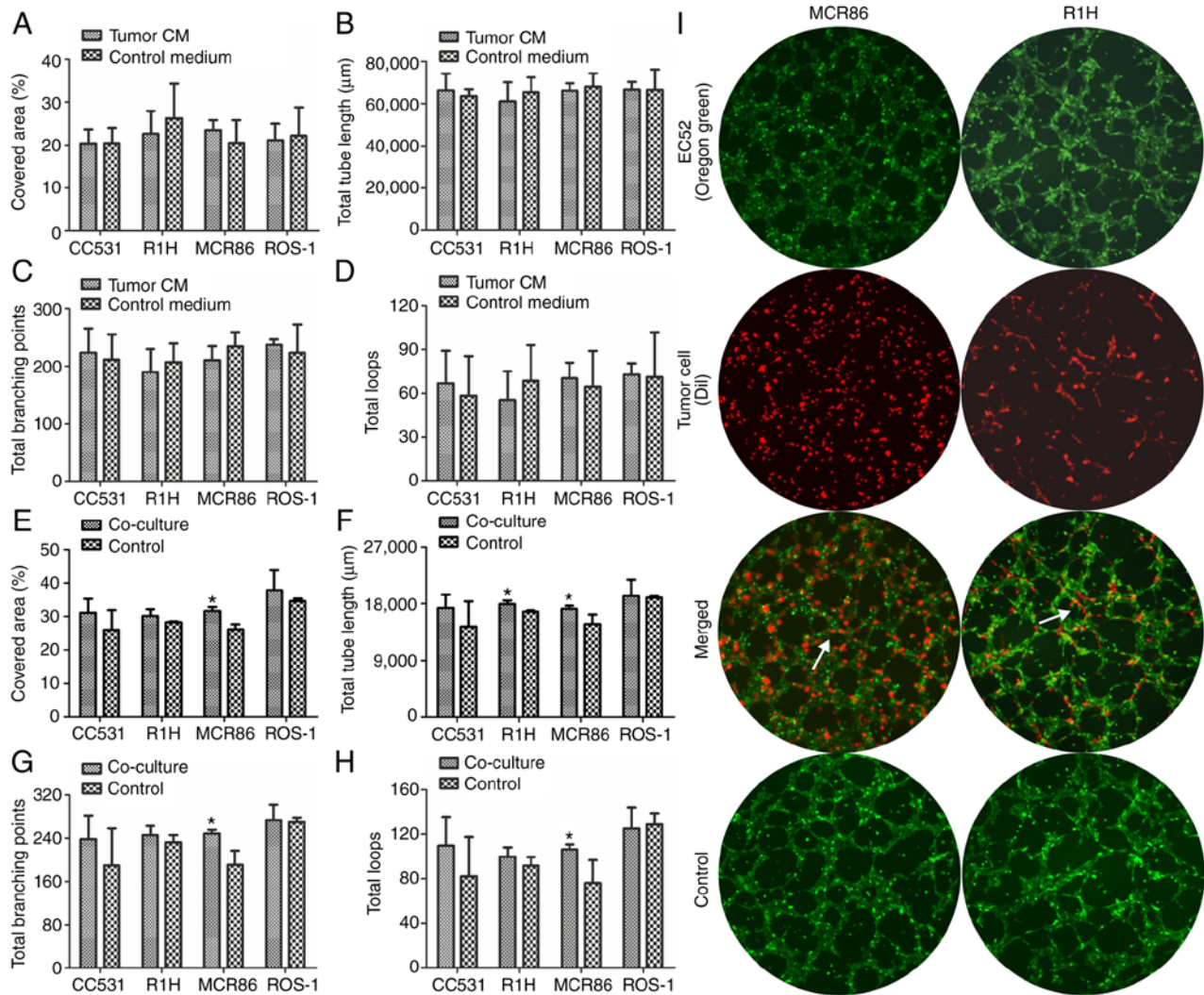


Figure 4. Tube formation of EC52 cells in tumor-CM and in direct co-culture with tumor cells. (A-D) Quantification of covered areas, total tube length, number of branching points, and total loops of tube formation generated by EC52 cells cultured in various tumor-CM and control medium. (E-H) Quantification of covered areas, total tube length, number of branching points, and total loops of tube formation generated by EC52 cells directly co-cultured with tumor cells. (I) Representative images of capillary-like tube structures generated by EC52 cells directly co-cultured with MCR86 and R1H tumor cells. DiI-labeled tumor cells are observed as red, and Oregon-green-labeled EC52 cells as green. The white arrows indicate the participation of tumor cells in tube formation. The results are expressed as the mean \pm standard deviation. * $P \leq 0.05$. CM, conditioned medium.

resistance and resistance to apoptosis (24). Similar findings were described in a study reporting on the direct co-culture of adipose-derived stem cells with breast cancer cells, indicating an exchange of cellular vesicles and fusion of cells, which led to a more malignant phenotype of the cancer cells (25). Hybrid cells derived from lung cancer cells and mesenchymal stem cells underwent an epithelial-mesenchymal transition (EMT), which resulted in a higher metastatic capacity and a higher expression of matrix metalloproteinase (MMP)2, MMP9 and typical mesenchymal markers, including vimentin (26). The authors suggest that this could be an explanation of the origin of lung cancer stem cells. A possible mechanism of the tumor-EC fusion was proposed by Mortensen *et al* (27), who demonstrated that, following intravascular dissemination, breast cancer cells fused with ECs in an *in vivo* mouse model. Hybrid cells exhibited markers of the two cell types and underwent mitosis. Although the present study did not further analyze cells in direct co-culture and drawing a distinct conclusion is therefore not possible, it was demonstrated that

tumor cells not only interact with ECs in a paracrine way, but also via direct cell-cell contact.

The presence of actively proliferating and functional ECs is essential for tumor angiogenesis (16). To clarify if and to which extent the various tumor cell lines contribute to the proliferation of ECs, EC52 cells were incubated in the CM of tumor cell lines and their viability was analyzed by a WST-8 assay. The breast cancer MCR86 and rhabdomyosarcoma R1H cells significantly stimulated the viability of the ECs. A previous study also detected a relatively low influence of tumor cells on the proliferation of endothelial progenitor cells (EPC), but a significant contribution to the recruitment and tube-like formation (28). Therefore, further experiments were performed focusing on the migration of ECs. As demonstrated by the WST-8 assay, the ECs were significantly affected by rhabdomyosarcoma and breast cancer cells. Their migration in the scratch test and the transmigration and invasion in the Boyden chamber assay were notably stimulated especially in the mammary

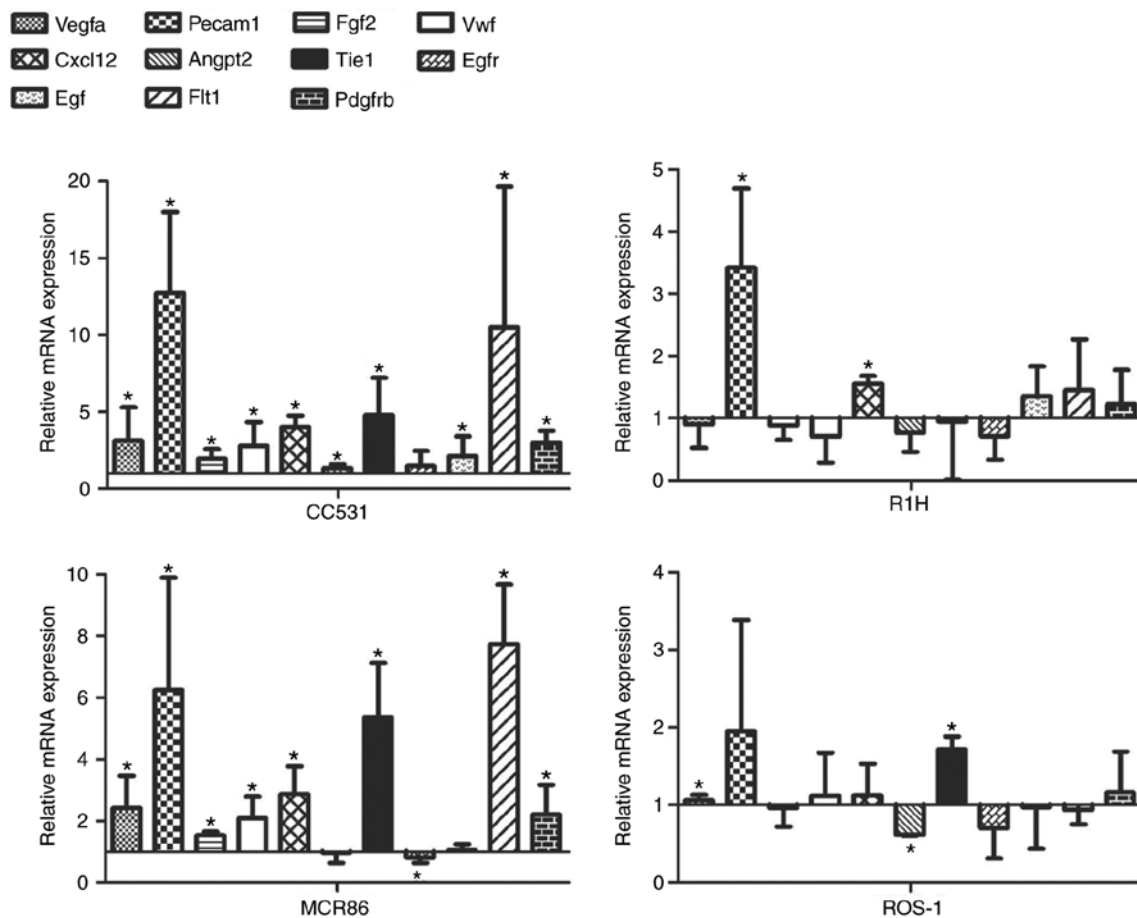


Figure 5. Effect of tumor-CM on gene expression in EC52 cells. Evaluation of the relative mRNA expression of 11 genes associated with angiogenesis and endothelial cell differentiation in EC52 cells following exposure to tumor-CM for 4 days compared with the control medium (the control data are normalized to 1). *P<0.05. CM, conditioned medium.

carcinoma CM. Ding *et al* (29) reported a positive correlation between increasing invasion and migration capacities and enhanced proliferation ability. This effect of the breast cancer cells appears not to be limited only to ECs, as they have also been demonstrated to activate an alteration in the morphology and migration properties of normal breast epithelial cells to trigger the EMT (21), and the chemotaxis of bone marrow-derived mesenchymal stromal cells (30).

The communication between ECs and other cell types is not only based on paracrine, but also on direct cell-cell interactions (28,31). Therefore, a co-culture system with the incubation of ECs in tumor-CM was assessed in a tube-formation assay. Tube formation is an *in vitro* model for evaluating the differentiation of ECs into tube-like structures, occurring subsequent to proliferation and migration (32). As anticipated from the aforementioned findings, the breast cancer cell line significantly stimulated the ECs to form tubes in the direct co-culture system. Furthermore, the tumor cells participated in the formation of tube-like structures (Fig. 4E-I). These findings support the hypothesis that tumor cells can modify their properties to form tumor blood vessels in terms of vasculogenic mimicry (28,33,34). However, the tumor-CM did not result in stimulating the EC52 cells to form capillary-like structures in the present study (Fig. 4A-D). This observation strengthens the hypothesis that tumor cells themselves contribute to tube formation

rather than further stimulating the tube-formation capability of ECs.

Contrary to the present findings, the CM of hepatocellular carcinoma and esophageal squamous cell carcinoma cells enhanced the tube-formation abilities of ECs (8,35). In another study, the CM from colon and breast cancer cells supported EC tube formation (36). On the one hand, these inconsistencies could be due to variances in cell lines, however they could also be explained by the different conditions of tumor-CM production. Possibly the concentration of tumor-derived pro-angiogenic factors in the CM produced in the present study is too low or the accumulation of metabolic waste products by tumor cells during the preparation of the CM is too high. On the other hand, since the effects of the tumor-CM in the other functional cell assays were notable, it is hypothesized that the tube formation in the present controls is itself high to the extent that a detectable increase is not possible.

Collectively, the present results demonstrated that breast cancer MCR86 cells exhibited the strongest potential to stimulate the angiogenic functions of ECs compared with the other assessed cancer types, supporting the clinical findings that invasive breast cancer belongs to the group of highly vascularized tumors and produces various angiogenic factors (37). The different effects observed among the tumor types can likely be linked to the soluble factors secreted by the tumor cells. In a previous study, the effect of these tumor cell lines on EPCs was

analyzed and the results suggested that tumor-derived cytokines, such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 2- α (MIP-2) and TNF-related apoptosis-inducing ligand (TRAIL), may serve a vital role in the cell-cell communications of tumor cells with their neighbors (28).

Given the contribution of tumor cells in changing the functional properties of ECs, the question of implicated gene expression changes arose. Genes associated with the phenotype of tumor-activated ECs may include the expression of the tumor-specific/induced markers of neovascularization (22). ECs isolated from human tumors exhibit enhanced angiogenic capabilities, survival, adhesion to tumor cells, chemotaxis and motility by expressing distinct markers to modulate their functions (3,38). *PECAM1* was upregulated in each group. This gene serves a key role in the initiation of vasculogenesis and angiogenesis, involving EC interactions on a cell-cell basis, but also with extracellular matrix components (39). *CXCL12* was significantly upregulated in the colon cancer CC531-, rhabdomyosarcoma R1H- and breast cancer MCR86-CM groups. This factor is typically expressed in stromal cells in various tissues and organs (40). However, it can interact synergistically with VEGF to promote the functions of vascular ECs, including cell migration, cell survival and changes in gene expression (41). *TIE1* levels were highest in the breast cancer MCR86-CM. Upregulation of *TIE1* has been observed in a variety of human solid tumors and tumor ECs, and is associated with tumor progression (42). The increase in the angiogenic capabilities of ECs following culture in tumor-CM could be partly attributed to these genes, based on the consistent upregulation in all the assessed groups.

VEGFA, *VWF*, *FGF2* and *PDGFRB* were revealed to be upregulated in the breast cancer MCR86- and colon cancer CC531-CM groups, and downregulated or unaffected in the osteosarcoma ROS-1- and rhabdomyosarcoma R1H-CM groups. The expression of VWF, an EC marker, is regulated by angiogenesis factors, such as FGF2 and VEGF. High VWF mRNA levels in tumors may be an early sign of activation of the endothelium (43). *PDGFR* serves a key role in regulating the formation and function of blood vessels (44). These gene expression results reflect the findings of the present functional assays. In nearly all the angiogenesis assays a stimulating effect of the breast cancer MCR86 cells was observed on the ECs. Furthermore, colon cancer CC531- and breast cancer MCR86-CM significantly induced EC migration. Conversely, CM derived from rhabdomyosarcoma R1H and osteosarcoma ROS-1, both categorized as sarcoma, induced nearly no significant changes in the functional assays or the expression of the majority of the tested genes, suggesting that the secretion of pro-angiogenic factors is likely dependent on the origin of the tumor cells.

Since it is widely reported that there is an association between vascularization density and disease prognosis for a number of carcinomas, but not sarcomas, this was evaluated by West *et al* (45) in 42 patients with high grade soft tissue sarcomas. The authors revealed no correlation between vascularization and metastasis or survival. In contrast, in invasive breast carcinoma, microvessel counts and density were associated with metastasis (46). Notably, Tomlinson *et al* (47) analyzed the angiogenesis patterns in sarcoma and carcinoma

samples and demonstrated that, in breast carcinoma tumors, the vessels were clustered within the stroma, whereas the vessels in the sarcoma stroma were homogeneously distributed. This could be due to the fibroblasts and myoblasts within the carcinoma stroma leading to a compartmentalization of the tumor, and thus of the angiogenic factors (47). In a review by Rocchi *et al* (48), the authors concluded that the angiogenesis in sarcomas could also serve an important role, but that there is an urgent requirement to analyze sarcoma neovascularization in greater detail. Based on the findings of the present study it can be concluded that the effect of carcinoma cells on angiogenesis is stronger than that of sarcoma cells. Whether this is associated with enhanced tumor progression or disease prognosis is to be evaluated in further *in vivo* studies. In the present study, rat tumor cell lines originating from tumors grown in the WAG/Rij rat were used. This allows using the same tumor cell lines in an established *in vivo* rat model for future studies making the translation from *in vitro* to *in vivo* much simpler. Further, using the WAG/Rij rat we can be certain that tumor cell lines used in the present study will grow *in vivo*. This would only be possible with human tumor cell lines when using an immunodeficient rat model with the disadvantage of having no immune system which is described to have an important effect for tumor growth and development.

Collectively, these results demonstrated that tumor-CM exerts a significant influence on the gene expression in ECs, leading to a modulation of signaling pathways that mediate the EC response. Significant upregulation of pro-angiogenic genes in ECs following culture in various tumor-CM is in agreement with the findings of enhanced angiogenic capacities of ECs in certain groups. The activation of quiescent ECs, which in turn secrete pro-angiogenic factors such as VEGF and FGF, can further lead to an autocrine and paracrine loop of angiogenesis stimulation (1). Therefore, it is reasonable to assume that tumor-CM exerts an effect on tumor angiogenesis by inducing the expression of pro-angiogenic genes in ECs. Previous findings demonstrated that CM derived from these various tumor cell types contain diverse secreted growth factors in varying amounts, capable of affecting the functional behavior of mature, but also of EPCs to different extents (28). Future studies should focus on which distinct factors, most likely among MCP-1, MIP-2, and TRAIL, serve a significant role in the interaction of tumor cells and mature ECs. Based on the present findings, the development of antibodies against these tumor cell-secreted molecules could provide a novel therapeutic option for treatment of highly vascularized tumors. Recently, a study by Kami Reddy *et al* (49) proposed the use of a novel inhibitor against dimethylarginine dimethylaminohydrolase 1 (DDAH1) for the treatment of prostate cancer. Inhibition of DDAH1 abrogated the secretion of angiogenic growth factors and reduced the vascularization of tumors, which led to an inhibition of xenograft tumors (49). Another approach to block the tumor-EC interaction is the inhibition of VEGFR1, which serves a role in tumor-associated, but not in adult physiological angiogenesis (50). Selective inhibition of this receptor would open up a new perspective in the treatment of highly vascularized tumors.

In summary, the present study demonstrated that tumor cells can significantly enhance the pro-angiogenic properties of ECs, including proliferation, migration, invasion and tube

formation, in a tumor-type-dependent manner, by influencing the genetic expression in ECs. Comparing the 4 distinct tumor types analyzed within this study, the breast cancer MCR86 cell line exhibited the greatest influence on the behavior of ECs. It is now the aim of future studies to evaluate the effectiveness of inhibiting tumor cell-secreted molecules to block the tumor-EC interaction in highly vascularized tumors.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MAA, RA, AMB, REH and AKW designed the study research and participated in the manuscript preparation, revision and data analysis. RS performed the quantitative polymerase chain reaction assays and data analysis. MW and MAA performed the proliferation and tube formation assays and the data analysis. AK assisted with the migration, transmigration and invasion assays and data analysis. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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