Gene expression and promoter methylation of angiogenic and lymphangiogenic factors as prognostic markers in melanoma

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The high mortality rate of melanoma is broadly associated with its metastatic potential. Tumor cell dissemination is strictly dependent on vascularization; therefore, angiogenesis and lymphangiogenesis play an essential role in metastasis. Hence, a better understanding of the players of tumor vascularization and establishing them as new molecular biomarkers might help to overcome the poor prognosis of melanoma patients. Here, we further characterized a linear murine model of melanoma progression and showed that the aggressiveness of melanoma cells is closely associated with high expression of angiogenic factors, such as Vegfc, Angpt2, and Six1, and that blockade of the vascular endothelial growth factor (VEGF) pathway by the inhibitor axitinib abrogates their tumorigenic potential in vitro and in the in vivo chicken chorioallantoic membrane assay. Furthermore, analysis of The Cancer Genome Atlas data revealed that the expression of the angiogenic factor ANGPT2 (P-value = 0.044) and the lymphangiogenic receptor VEGFR-3 (P-value = 0.002) were independent prognostic factors of overall survival in melanoma patients. Enhanced reduced representation bisulfite sequencing-based methyleone profiling revealed for the first time a link between abnormal VEGFC, ANGPT2, and SIX1 gene expression and promoter hypomethylation in melanoma cells. In patients, VEGFC (P-value = 0.031), ANGPT2 (P-value < 0.001), and SIX1 (P-value = 0.009) promoter hypomethylation were independent prognostic factors of shorter overall survival. Hence, our data suggest that these angio- and lymphangiogenesis factors are potential biomarkers of melanoma prognosis. Moreover, these findings strongly support the applicability of our melanoma progression model to unravel new biomarkers for this aggressive human disease.

Abbreviations
ANGPT, angioipoietin; CAM, chicken chorioallantoic membrane; FGF, fibroblast growth factor; IRS, immunoreactive score; PDGF, placental growth factor; TCGA, The Cancer Genome Atlas; VEGFR, vascular endothelial growth factor receptor; VEGF, vascular endothelial growth factor.
1. Introduction

Melanoma mortality rate, one of the highest among all human cancers, is broadly associated with its metastatic potential (Timár et al., 2016). Although the complete resection of localized melanomas is curative in nearly all cases (Shain and Bastian, 2016), the survival rate for patients identified with metastatic melanoma is only 6–11 months (Clark et al., 2018; Fruehauf et al., 2011).

Metastasis is a complex process, in which a sufficient blood supply is critical for dissemination and subsequent tumor growth. Melanoma cells can spread through hematogenous and lymphatic routes; therefore, the formation of new blood and lymphatic vessels via angiogenesis and lymphangiogenesis, respectively, is crucial (Adler et al., 2017). Indeed, high vascularization has been associated with melanoma progression (Chung and Mahalingam, 2014; Timár et al., 2016).

Melanoma cells produce several proangiogenic factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), placental growth factor (PDGF), and angiopoietin (ANGPT), which are involved in tumor vascularization (Matsumoto and Claesson-Welsh, 2001). One of the major players is the VEGF family, which is comprised of the glycoproteins VEGFA, VEGFB, VEGFC, VEGFD, and PDGF, and their tyrosine kinase receptors vascular endothelial growth factor receptor (VEGFR)-1 (FLT1), VEGFR-2 (KDR), VEGFR-3 (FLT4), and co-receptor NRP. The VEGFA signaling pathway is classically known to induce angiogenesis by the activation of VEGFR-2, while VEGF-3, activated by VEGFC and VEGFD, is specifically related to lymphangiogenesis (Matsumoto and Claesson-Welsh, 2001). Notably, cutaneous melanomas have a high metastatic potential to lymph nodes, which is associated with high expression of VEGFC (Adler et al., 2017; Boone et al., 2008).

As mentioned, diverse molecules are involved in vessel formation. VEGFs are associated with the early steps of blood vessels formation, while ANGPTs are critical regulators of vascular and lymphatic maturation and remodeling (Rigamonti et al., 2014; Thurston, 2003). ANGPT1 and ANGPT2 bind to the tyrosine kinase receptor TIE2; the former is considered an agonist and the latter primarily an antagonist of this receptor. ANGPT2 has been shown to be highly expressed in tumors, promoting endothelial disruption and facilitating tumor cell extravasation (Li et al., 2015).

Angiogenic factors have been shown to be useful in predicting cancer progression and aggressiveness in different malignancies and were proposed as tumor biomarkers (Cao et al., 2014; Martinek et al., 2013). Studies with melanoma patients show conflicting results; while some have shown that VEGFA and VEGFC can predict shorter overall and disease-free survival (Boone et al., 2008; Spiric et al., 2015; Tas et al., 2006), other studies failed to prove such a correlation (Bolander et al., 2007; Vihinen et al., 2007). Recently, it has been reported that the promoter regions of the major angiogenesis players contain extended CpG islands (Pirola et al., 2018). Thus, DNA methylation might be a potent regulatory mechanism of angiogenesis in melanoma.

Here, we show that the aggressiveness of murine melanoma cells is closely associated with high expression of angiogenic factors and that blockade of the VEGF pathway abrogates the tumorigenic potential of metastatic melanoma cells. Furthermore, the expression of the angiogenic factor ANGPT2 and the receptor VEGFR-3 were significantly associated with overall survival of melanoma patients. The methylation status of VEGFC, ANGPT2, and SIX homeobox 1 (SIX1) promoters was also found to correlate with overall survival in melanoma patients. In the studied murine model, DNA methylation was identified as the mechanism regulating the abnormal expression of these genes.

2. Methods

2.1. Cell lines and drug treatment

Murine melanoma cell lines 4C11− (nonmetastatic) and 4C11+ (metastatic) were cultured in RPMI 1640 medium supplemented with 5% FBS and 1% penicillin (100U⋅mL⁻¹) and streptomycin (100 μg⋅mL⁻¹) at 37 °C in 5% CO₂ humidified atmosphere. Cell culture reagents were purchased from PAN Biotech (Aidenbach, Germany). Axitinib (PZ0193; Sigma-Aldrich, St. Louis, MO, USA), a selective inhibitor of VEGF receptors, and 5-Aza-2’-deoxycytidine (5-Aza-CdR; Calbiochem, Merck, Darmstadt, Germany) were dissolved in DMSO (PAN Biotech) and stored at a final concentration of 10 mM at −20 °C. 4C11+ cells were treated with different concentrations (40 nm-10 μM) of axitinib for MTT assay and with 1 μM for all other assays. All treatments were performed for 48 h. 4C11− cells were treated with 10 μM of 5-Aza-CdR for 72 h. As a control, cells were treated with the respective volume of DMSO. Final DMSO volume in the cell culture was lower than 0.01%.

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2.2. In vivo chicken chorioallantoic membrane assay

The chicken chorioallantoic membrane (CAM) assay was performed as previously described (Muenzner et al., 2018). Fertilized specific pathogen-free chicken eggs were obtained from Valo BioMedia (Osterholz-Scharmbeck, Germany) and incubated at 37 °C with ~80% relative humidity. The first day of incubation was considered as embryonic day (EDD) 1. On EDD 8, the eggshell was opened at the more rounded pole of the egg and the exposed membrane residing below the air sac was removed with fine forceps revealing the CAM, and the window was re-sealed with adhesive silk tape. On EDD 9, 4C11- and 4C11+ cells or pretreated 4C11+ cells (axitinib or vehicle) (1 × 10⁶ cells/egg) were applied on the CAM. Cells were prepared in a mixture of 50% RPMI medium and 50% Matrigel (Corning® Matrigel® Basement Membrane Matrix, 356237; Corning, Bedford, MA, USA), and the formed pellets were incubated for 1 h at 37 °C before being applied onto the CAM. Tumors and the adjacent CAM were dissected on EDD 12 or EDD 15. Tumor volume was measured (l × w × h × 0.526, where l indicates length, w indicates width, and h indicates height), and the tissue was fixed in 4% phosphate-buffered formaldehyde before being embedded in paraffin for histopathologic observation. After the tumor and CAM had been removed, the embryo was immediately euthanized by decapitation.

2.3. Immunostaining

Immunohistochemistry (IHC) was performed to detect pH3 [phospho-histone H3 (BC37), 1 : 200; Biocare Medical, Pacheco, CA, USA] in formalin-fixed paraffin-embedded (FFPE) tissue obtained from the CAM assay (n = 7); and VEGFR-3 [(D6), 1 : 200; Santa Cruz, Dallas, TX, USA] and ANGPT2 [(F-1), 1 : 100; Santa Cruz] in primary and metastatic FFPE human melanoma specimens (n = 5). The study methodologies conformed to the standards set by the Declaration of Helsinki. Briefly, sections (2–4 μm) were deparaffinized at 72 °C for 30 min, incubated in xylene, and rehydrated in EtOH. Antigen was retrieved by heating in a Tris/EDTA buffer at 120 °C for 5 min, and endogenous peroxidases and nonspecific binding sites were blocked with specific blocking solution. The slices were incubated with specific primary antibody and next with secondary horseradish peroxidase-linked antibody. Positive immunoreactivity was detected using diaminobenzidine or AEC/H₂O₂, and nuclei were counterstained with hematoxylin and eosin (HE). Immunoreactive score (IRS) was assessed as described previously (Dumitru et al., 2013).

Hematoxylin and eosin-stained tumor slices obtained from the CAM assay were scanned with Panoramic MIDI system (Camera type: CIS VCC-FC60FR19CL, Objective name: Plan-Apochromat, Objective magnification: 40×, Camera adapter magnification: 1×) (3DHISTECH, Budapest, Hungary), which generates a digital image with high quality, and evaluated with the CASEVIEWER software (Version 2.0; 3DHISTECH).

Immunofluorescence images of cryosections of primary (n = 3) and metastatic (n = 3) melanoma were generated using the multi-epitope ligand cartography technique (MELC). Sample preparations from tissue, data generation, and analysis were performed as described previously (Ostalecki et al., 2017). The following antibodies were used: ANGPT2-Alexa Fluor 488 [(MM0020-1F29), 1 : 40; Novus Biologicals, Centennial, CO, USA], VEGFR-3-PE [(9D9F9), 1 : 20; BioLegend, San Diego, CA, USA], and Collagen IV-FITC [(5K134), 1 : 200; Biomol, Hamburg, Germany).

2.4. Total RNA isolation

Total RNA from 4C11− and 4C11+ melanoma cells was prepared using the miRNeasy Mini kit (Qiagen, Hilden, Germany), and DNA digestion was performed with RNase-Free DNase Set (Qiagen) according to the manufacturer’s protocol.

2.5. NanoString gene expression analysis

mRNA signature of 4C11− and 4C11+ cancer cells was profiled with nCounter PanCancer Mouse Pathways Panel (NanoString Technologies, Seattle, WA, USA). As recommended by the manufacturer, 100 ng of total RNA was used as input for sample preparation. Samples and specific probes were hybridized overnight at 65 °C, automatically processed in the Prep Station, and transferred to the Digital Analyzer for data collection with a high-density scan (600 fields of view). NanoString nSolver software was used for normalization and pairwise comparisons. mRNA data were normalized with a set of 20 predetermined housekeeping genes. Based on background detection, the minimal threshold for detection was considered as 50 counts.

2.6. RT-qPCR

Following the manufacturer’s instructions, 1 μg of purified RNA was reverse transcribed to cDNA using miScript® II RT kit (Qiagen) with HiFlex Buffer.
Real-time PCR was performed with QuantiTect SYBR® Green PCR Kit (Qiagen) using 40 ng of cDNA and specific primers designed with NCBI Primer-Blast software (Table S1). The PCR amplification conditions were as follows: 95 °C for 15 min, 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 70 °C for 30 s. Beta-actin was used as a housekeeping control, and data were analyzed with the comparative 2−ΔΔCT method. PCR reactions were performed using the BioRad CFX96 Real-Time PCR Detection System.

2.7. Invasion assay
Membranes of Transwell® inserts (Corning) were pre-coated with Matrigel Growth Factor Reduced (356231; Corning) diluted in serum-free medium in a 1 : 4 proportion. After Matrigel had jellified, 2 × 10⁵ previously serum-starved (24 h) cells were seeded in the upper chamber in serum-free medium. RPMI containing 10% FBS was used as a chemoattractant in the lower chamber. Cells were allowed to invade for 48 h at 37 °C and 5% CO₂. After this period, membranes were fixed with 4% formaldehyde and stained with crystal violet. Images of invading cells were obtained in a bright-field microscope (Leica DMi1; Leica Microsystems, Wetzlar, Germany).

2.8. Tube formation assay
A 96-well plate was precooled, and 40 μL of Matrigel® (356237; Corning) was added in each well. The plate was incubated at 37 °C for at least 30 min to allow the Matrigel® to form a gel-like structure before 1–6 × 10⁴ cells were seeded on top of the Matrigel® in 100 μL of complete medium. Tube formation was assessed after 16–18 h under a bright-field microscope (Leica DMi1).

2.9. MTT assay
Four thousand 4C11+ cells were seeded in the wells of a 96-well plate and allowed to attach for 24 h. Following this, cells were treated with different concentrations of axitinib (Sigma-Aldrich, Darmstadt, Germany) for 48 h and cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. For this, cells were incubated with MTT (Sigma-Aldrich, Germany) for 48 h and cell viability was analyzed by MTT. Beta-actin was used as a housekeeping control, and data were analyzed with the comparative 2−ΔΔCT method. PCR reactions were performed using the BioRad CFX96 Real-Time PCR Detection System.

2.10. Wound healing assay
Cells were seeded in a 12-well plate, were led to adhere for 24 h, and then treated with axitinib or vehicle alone for 48 h. At this point, the monolayer confluence was reached and a straight wound was created with a sterile 100 μL pipette tip in the center of each well and cell debris were eliminated by washing with PBS. Cells were incubated with FBS-free medium containing 2 ng·μL⁻¹ of mitomycin C (Sigma-Aldrich), a mitosis inhibitor, and cell migration was monitored for 48 h by bright-field microscopy (Sigma-Aldrich, Tokyo, Japan). The cell-free area was measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA), and relative cell migration was quantified by the equation: Migration % = [1 – (cell-free area at t₄₈/cell-free area at t₀)] × 100.

2.11. Cell cycle analysis
For cell cycle analysis, after the appointed treatment, adherent and occasional floating cells in the supernatant were collected, washed in PBS, and fixed over-night with ice-cold 70% EtOH. Next, cells were incubated with staining solution containing 50 μg·mL⁻¹ propidium iodide and 0.5 mg·mL⁻¹ RNase A for 30 min in the dark. DNA content was determined in FACSCanto® II flow cytometer (Becton-Dickinson, San Diego, CA, USA), and cell cycle distribution was assessed with FLOWJO cell cycle platform v7.6.5 (Flowjo LCC, Ashland, OR, USA).

2.12. Bioinformatic analysis
Pathway enrichment analysis was performed using the Panther 2016 database in Enrichr domain (http://amp.pharm.mssm.edu/Enrichr). Pathways with P < 0.05 were considered as significant. Protein interactions were analyzed using STRING (https://string-db.org/).

2.13. Survival analysis
Data on gene expression and methylation of 470 individuals were downloaded from The Cancer Genome Atlas (TCGA) Skin Cutaneous Melanoma project. Of these, 79 individuals bearing primary tumors and 199 bearing secondary tumors (totalizing 278) had information on all covariates considered in this study and were used for the survival analyses. A multivariate Cox regression model was used to test the impact of gene expression and promoter methylation on patient overall survival. Age, tumor primary site, presence of metastasis in lymph nodes, ulceration, and Breslow
depth value were used as covariates. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) are shown. Statistical significance was set at \( P < 0.05 \). Kaplan–Meier survival curves were generated for genes with significant association with overall survival.

2.14. Statistical analysis

Data analysis was performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). Two and multiple group comparisons were analyzed by \( t \)-test and ANOVA, respectively. For non-normally distributed data, the Mann–Whitney test was used for two-group comparison.

3. Results

3.1. Melanoma cell line 4C11+ exhibits highly aggressive and angiogenic phenotype in vitro and in vivo

4C11− and 4C11+ cells were engrafted onto the CAM and incubated for 3–5 days. 4C11- tumors grew at a slow rate, with only a small tumor mass being visible at day 3. However, 4C11+ cells at day 3 had already given rise to a tumor mass with an extensive network of blood vessels. At day 5, most of the embryos bearing 4C11+ tumors had died, probably due to the tumor aggressiveness. The 4C11+ tumors of chicken embryos that survived until day 5 were also removed and analyzed by HE staining; however, these samples harbored large hemorrhagic areas leaving hardly any tumor tissue (Fig. S1), which prevented further investigations. Thus, we decided to exploit 4C11− tumors on day 5 and 4C11+ tumors on day 3. In average, 4C11+ tumor volume was 11-fold bigger than 4C11− tumors at the evaluated time points (Fig. 1A). For objective evaluation, HE-stained tumor slices were scanned, which allowed the measurement of the actual tumor area by considering only tumor cell regions and excluding residual Matrigel®, CAM tissue, and large hemorrhagic areas. In average, the 4C11+ tumor area was 5-fold bigger than the area of 4C11− tumors (Fig. 1B).

Hematoxylin and eosin histological analysis showed that in 4C11− tumors, neoplastic cells were separated by profuse eosinophilic material, such as Matrigel®. In these tumors, we found isolated intervening vessels and no evidence of CAM invasion (Fig. 1C, left panels). In contrast, 4C11+ tumors presented hyperchromatic nuclei, pleomorphic cells, extensive necrosis, and a hypervascularized and invasive tumor growth pattern (Fig. 1C, right panels). There was also a variable pigment formation in 4C11+ samples, which was clearly recognizable in the macroscopic tumor and in the HE staining. Abundant positive immunohistochemical staining for pH3, an important mitosis marker, was observed in 4C11+ cells (Fig. 1C, right panel). Staining quantification showed that 4C11+ tumors had in average 3 times more pH3-positive cells [31/high-power field (HPF); range 20–45] than 4C11− tumors (9/HPF; range 3–15; Fig. 1D), confirming the high proliferative index of 4C11+ cells (Fig. 1C). In addition, 4C11+ cells were shown to be highly invasive in vitro (Fig. 1E).

Scanned slices were also employed to better analyze tumor vascularization. All 4C11− tumor samples contained few and well-defined intratumoral vessels; however, 4C11+ tumor samples presented large and irregular vessels and several hemorrhagic areas within the tumor masses (Fig. 1F). From seven analyzed tumors established from 4C11+ cells, four had large areas of hemorrhage and one was completely hemorrhagic (Fig. 1F, bottom right panel). In a tube formation assay in vitro, we could observe that 4C11+ cells formed an extensive network of capillary-like structures, while 4C11− cells remained as single cells or formed irregularly shaped clusters of cells, and were, therefore, incapable to generate defined tubular vasculogenic mimicry (Fig. 1G).

3.2. Transcriptional analysis reveals alteration in angiogenesis-related genes during melanoma progression

To explore the molecular mechanisms responsible for the highly aggressive phenotype of 4C11+ cells, we profiled the expression pattern of 770 murine cancer-related genes in 4C11− and 4C11+ cells with the NanoString nCounter technology. Using a twofold difference and \( P < 0.05 \) cutoff, we found 254 differentially expressed mRNA, of which 59 were significantly upregulated and 195 downregulated in 4C11+ in comparison with 4C11− cells (Fig. 2A and Tables S2 and S3). Unsupervised hierarchical clustering analysis demonstrated that 4C11− and 4C11+ cells could be distinguished accordingly to their mRNA expression profile (not shown). The 10 most up- and 10 most downregulated genes were hierarchically clustered and displayed in a heatmap (Fig. 2B). To gain further insight into the function of the dysregulated genes in 4C11+ cells, we performed a pathway enrichment analysis of all dysregulated genes using the Panther database. Among the most significant enriched pathways were FGF, PDGF, and VEGF signaling pathways, all...
Fig. 1. Melanoma cell line 4C11+ exhibits aggressive and proangiogenic phenotype in vivo. (A) Volume of tumors obtained in CAM assay. (B) Tumor area was quantified in Case Viewer software. Only tumor cells were quantified. Matrigel® and occasional blank spaces (cutting artifacts) were excluded from the total tumor area. (C) 4C11− and 4C11+ cells mixed with Matrigel® were applied on the CAM and incubated for 5 and 3 days, respectively. Representative macroscopic images, HE (4× and 40× magnification) and pH3 (40× magnification)-stained sections of 4C11− and 4C11+ tumors are shown. In the 4C11− tumor ex ovo image, arrows highlight the small tumor. In HE images, arrows indicate blood vessels and arrowheads indicate mitotic cells. Each group consisted of at least seven specimens. (D) Quantification of pH3-positive staining per HPF in CAM assay samples. (E) Representative images of Transwell invasion assay of 4C11− and 4C11+ cells after 48-h incubation. Scale bar: 100 μm. (F) HE-stained sections of 4C11− (3 days) and 4C11+ (5 days) tumors grown on the CAM were visualized by the CASE VIEWER software. Arrows and asterisks indicate blood vessels and hemorrhagic areas, respectively. Left panels show 4C11− tumors, which present well-defined tumor vessels. Right panels show 4C11+ tumors, and the top right depicts extravasated blood cells adjacent to melanoma cells, while bottom right illustrates a large hemorrhagic area surrounding tumor cells. Scale bar: 50 μm. (G) Tube formation capability was assessed for 4C11− and 4C11+ cells seeded on Matrigel® after incubation of 16 h. Scale bar: 250 μm. Data are shown as mean ± SD, and statistical analysis was performed with Mann–Whitney U-test (A) or t-test (B-C) (**P ≤ 0.001; **** P ≤ 0.0001).
directly associated with tumor progression and, most importantly, tumor metastasis and angiogenesis (Fig. 2C). KEGG pathway analysis also showed enrichment of tumor-associated pathways (not shown). Interestingly, a String analysis showed that among the 10 most upregulated genes in 4C11+ compared with 4C11− cells, there is an association among the proteins encoded by Vegfc (the most upregulated gene), Angpt2, She4, and Met. The same analysis with the top 10 downregulated genes depicted an interaction network among Tsc, Fgfr2, Cola1a, Bmp4, Bmp7, Lef1, and Gpc4 (Fig. 2E).

These data, in association with our functional assays, prompted us to study the angiogenesis process in these cells. Moreover, as Vegfc was the most dysregulated gene in the NanoString analysis, we decided to evaluate its family in more detail. We confirmed the differential expression of Vegfa, Vegfb, Vegfc, and their receptors, Vegfr-1, Vegfr-2, Vegfr-3, and Nrp2 by RT-qPCR (Fig. 2E,F). We validated that Vegfa is downregulated in 4C11+ cells in comparison with 4C11− cells, while Vegfb and Vegfc are upregulated, the latter to a much higher extent. Considering the receptors, Vegfr-1 and Nrp2 were downregulated in 4C11+ cells compared with 4C11− cells; Vegfr-2 could not be detected in either one of the cell lines and Vegfr-3 was highly upregulated in 4C11+ cells. Additionally, we confirmed the higher expression of Angpt2, Met, and Six1 in 4C11+ cells (Fig. 2G).

3.3. Inhibition of VEGFC function decreases aggressiveness phenotype of metastatic 4C11+ cell line

To better understand the impact of VEGFC in the aggressiveness of 4C11+ cells, we blocked the VEGFC pathway using axitinib, a potent and selective inhibitor of VEGF receptors. First, we evaluated the effects of axitinib on 4C11+ cells viability by a MTT dose-response analysis. Cells were treated with 40 nm–10 μm of axitinib for 48 h. Growth inhibition obtained using 1–5 μm of the drug was similar, ~40%, while 10 μm of the drug reduced cell viability to nearly 50%. For this, we decided to use the lowest effective dose of axitinib in 4C11+ cells, that is, 1 μm, for the subsequent experiments (Fig. 3A). Cell cycle analysis revealed that axitinib treatment induced a significant decrease of cells in the G1 phase, while massively increasing the G2/M population (13.4% in control cells to 52.2% in treated cells). There was no alteration in the sub-G1 population (Fig. 3B). In wound healing assays, vehicle-treated 4C11+ cells were able to close the wound after 24 h; however, axitinib-treated cells had a 30% lower migration rate at that time point (Fig. 3C). After 48 h, there was no significant change in migration capability (data not shown). In the in vitro CAM assay, we observed that vehicle-treated 4C11+ cells were able to develop massive tumors in 5 days, which were extremely invasive and presented extensive inflammatory infiltrates (Fig. 3D, left panels). On the other hand, axitinib-treated 4C11+ cells generated only small tumor masses, in some cases failing to develop a tumor mass at all. HE staining depicts tumor cells surrounded by Matrigel® and clearly separated from the CAM, which was not infiltrated by the tumor cells (Fig. 3D, right panels). From 11 samples grown from axitinib-pretreated 4C11+ cells, two were able to develop tumors, although much smaller than tumors generated by untreated 4C11+ cells. In one graft, cells were largely surrounded by Matrigel®, which was not observed in the control group, while in the other graft, the tumor growth was very similar to the DMSO-treated group with cells invading the CAM and presenting high immune cell infiltration (Fig. S2).

3.4. High expression of VEGFR-3 and ANGPT2 is associated with poor overall survival in melanoma patients

Cox multivariate analysis was performed to evaluate the association of gene expression and overall survival in melanoma patients. Expression of the following genes detected as upregulated in 4C11+ cells in comparison with 4C11− cells was analyzed individually: VEGFC, VEGFR-3, ANGPT2, MET, and SIX1. After adjustment for age, tumor primary site, presence of metastasis in lymph nodes, ulceration, and Breslow depth value, high expression of VEGFR-3 (HR = 1.199; P-value = 0.044) and ANGPT2 (HR = 1.189; P-value = 0.002) was shown to be predictors of shorter overall survival (Fig. 4A). Kaplan–Meier curves of VEGFR-3 and ANGPT2 are shown (Fig. 4B,C). The expression of VEGFR-3 and ANGPT2 was also evaluated by IHC and MELC staining in primary and metastatic human melanoma tissue. In the IHC, only the staining of melanocytes and melanoma cells were evaluated. Skin and colon tissue were used as negative controls (Fig. S3). The average positive staining intensity of the antigens was weak in primary samples (IRS: VEGFR-3 = 3.6; ANGPT2 = 3.1) and moderate in the metastatic specimens (IRS: VEGFR-3 = 5.3; ANGPT2 = 5.6; Fig. 4D). In the MELC technique, we also stained blood vessels with Collagen type IV. This assay confirmed the high expression of ANGPT2 and VEGFR-3 in metastatic melanomas and showed these antigens
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C. Pathways

Angiogenesis
FGF signaling pathway
Apoptosis signaling pathway
Integrin signaling pathway
p53 pathway
EGF receptor signaling pathway
PDGF signaling pathway
CCP signaling
Ras Pathway
VEGF signaling pathway

D. Up-regulated genes

Down-regulated genes

E.

F.

G.

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Fig. 2. Transcriptional analysis reveals alteration in angiogenesis-related genes during melanoma progression. (A) Scatter plot of 4C11– and 4C11+ mRNA expression assessed by the NanoString PanCancer Mouse Pathways Panel (n = 3). Each dot represents one gene. (B) Heatmap of unsupervised hierarchical clustering of 4C11– and 4C11+ cells with the 10 most upregulated and 10 most downregulated mRNA. Green and red represent, respectively, low and high mRNA expression level. (C) Ten most significantly enriched pathways of all dysregulated genes determined by the Panther database. (D) String analysis of the 10 most upregulated and downregulated genes in 4C11+ mRNA. Green and red represent, respectively, low and high mRNA expression level. (E–G) Vegfa, Vegfb, Vegfc (E); Vegfr-1, Vegfr-2, Vegfr-3, Npr2 (F); and Angpt2, Met, and Six1 (G) mRNA expression was determined by RT-qPCR in 4C11– and 4C11+ cells. Data are expressed as fold change normalized to Actb (n = 3). nd: not detected. Data represent mean ± SD, and statistical significance was evaluated by one-way ANOVA followed by the Dunnett’s post hoc test (**P ≤ 0.01; ***P ≤ 0.001).

are expressed by both the tumor and endothelial cells. While VEGFR-3 was mainly detected in the tumor vasculature, ANGPT2 was highly expressed by the tumor cells (Fig. 4E).

3.5. Expression of Vegfc, Angpt2, and Six1 is epigenetically regulated in murine melanoma cell lines

The DNA methylation status of Vegfc, Vegfr-3, Angpt2, Met, and Six1 was verified in a methylome sequencing data from enhanced reduced representation bisulfite sequencing (Rius et al., in preparation). CpGs distant up to 1500 nucleotides upstream and 250 nucleotides downstream from the transcription start site (TSS) were analyzed, and a CpG site was considered to be differentially methylated if it presented a minimum of 25% difference in methylation with a P-value ≤ 0.01. The number of CpGs differentially methylated in Vegfc, Angpt2, and Six1 promoters was four (distant from 147–222 nucleotides downstream of the TSS), 11 (located from 1129 to 1370 nucleotides upstream of the TSS), and 72 (–1499 to +203 distant from the TSS), respectively. These CpGs sites had in average 33%, 87%, and 76% lower methylation in 4C11+ cells than in 4C11– cells, respectively (Fig. 5A and Table S4). In the analyzed region, Vegfr-3 and Met did not show any differential methylation in 4C11+ in comparison with 4C11– cells. We then treated 4C11– cells, which express low levels of Vegfc, Angpt2, and Six1, with the DNA methyltransferase inhibitor 5-Aza-CdR and verified their expression by RT-qPCR. Demethylation caused by the treatment enhanced the expression of the three genes analyzed, most prominently of Angpt2 and Six1 (Fig. 5B).

3.6. Promoter methylation of VEGFC, ANGPT2, and SIX1 is associated with poor prognosis in melanoma patients

As we observed an epigenetic regulation of Vegfc, Angpt2, and Six1 in our mouse melanoma model, we analyzed whether the promoter methylation of these genes could predict overall survival in melanoma patients by a Cox multivariate analysis. Promoter CpG island and shore methylation of VEGFC (HRisland = 0.035; P-value = 0.031) and SIX1 (HRisland = 0.591; P-value = 0.009) were analyzed and found to be significantly associated with survival. ANGPT2 promoter does not contain a CpG island; therefore, we analyzed single CpGs shown to be hypomethylated in 4C11+ cells and that had also been previously evaluated in chronic lymphocytic leukemia (Martinelli et al., 2013). The average DNA methylation of these CpGs was associated with overall survival (HR = 0.1677; P-value < 0.001). In all cases, decreased methylation was associated with shorter survival of melanoma patients (Fig. 5C). Kaplan–Meier curves are shown (Fig. 5D–F).

4. Discussion

Metastasis is closely associated with high mortality rate in melanoma patients. Therefore, melanoma cell dissemination and the related molecular mechanisms still need to be elucidated in more detail. New diagnostic and prognostic markers are also important to reach a better clinical outcome and mortality reduction. To study melanoma progression, we used a linear murine progression model in which metastatic 4C11+ cells arose from nonmetastatic 4C11– cells following P53 expression loss (Souza et al., 2012).

To better analyze the cancer properties of 4C11– and 4C11+ cells, we performed the CAM assay, which allows the study of several hallmarks of cancer, as proliferation, invasion, metastasis, and angiogenesis (Lokman et al., 2012; Muenzner et al., 2018). Here, we could observe a clear distinction between the two cell lines. In vivo, 4C11+ cells gave rise to larger tumors, which were highly proliferative and vascularized. On the other hand, 4C11– tumors displayed small well-defined tumor masses with localized tumor vessel infiltration. We also observed 4C11+ cells were highly invasive in vitro. These data are consistent and enrich our previous results showing different growth rate and metastasis capability of 4C11– and 4C11+ cells in a
mouse model (Souza et al., 2012). Interestingly, 4C11+ cells also developed an extensive network of capillary-like structures in an in vitro tube formation assay, indicating a high vascular mimicry (VM) capacity. These 3D tube-like structures consist of tumor cells and extracellular matrix and are endothelial cell-free. VM can function as an alternative supplier of blood to tumor masses, independently of angiogenesis, and can contribute to metastasis (Chung and Mahalingam, 2014). The high VM capability of 4C11+ cells is...
consistent with their excessive bleeding observed in the CAM assay and can contribute to it.

Investigation of the expression pattern of cancer-related genes in 4C11− and 4C11+ cells revealed that Vegfc and Angpt2 were among the top 10 upregulated genes. Both are important regulators of the vascular phenotype in tumors (Kim et al., 2009). A pathway-based analysis of all dysregulated genes revealed enrichment of several angiogenic pathways, including FGF, PDGF, and VEGF signaling. In String analysis, we found that VEGFC, ANGPT2, MET, and SHC4 proteins interact, which is a further support of their possible role in the 4C11+ cells aggressive phenotype. Several downregulated genes were shown to have a strong protein interaction, but most of these genes, such as Fgfr2, Tnc, and Lef1, have been positively associated with tumor progression (Katoh and Nakagama, 2013; Murakami et al., 2001; Shao et al., 2015), suggesting their downregulation is not involved in 4C11+ cells aggressive phenotype. Conversely, Bmp4 is a known inhibitor of angiogenesis (Tsuchida et al., 2014); thus, its downregulation probably contributes to 4C11+ tumor vascularization. Compared with 4C11− cells, 4C11+ cells presented upregulation of
Vegfb, Vegfc, and Vegfr-3. Importantly, the expression of VEGF receptors by tumor cells is a known indicator of high aggressiveness (Mouawad et al., 2009). As VEGFC has a high affinity for VEGFR-3, our data indicate that the VEGF pathway effector in 4C11+ cells must be VEGFC by binding and activating VEGFR-3 in an autocrine signaling, which is classically recognized to be involved in lymphangiogenesis (Mouawad et al., 2009).

As oxygen and nutrient supply are mandatory for tumor growth, the VEGF pathway, angiogenesis, and lymphangiogenesis are not only essential for the metastasis process, but also for the progression of solid tumors beyond a critical size (Alitalo et al., 2013). Indeed, recently VEGFC and VEGFD have also been reported to regulate the inflammatory tumor microenvironment, which regulates early stages of tumor growth (Alitalo et al., 2013). Nonetheless, high levels of VEGFC correlate with melanoma metastasis to lymph nodes, which is one of the most important markers of poor prognosis for melanoma patients (Timár et al., 2016). It has also been demonstrated that VEGFC and lymphangiogenesis can contribute to the development of distant metastasis (Ma et al., 2018).

We also confirmed the expression of Angpt2, Met, and Six1; genes involved in metastasis and angiogenesis. ANGPT2 is a secreted growth factor that sensitizes endothelial cells to different proangiogenic factors, such as VEGFs, and it has been shown to promote tumor metastasis, angiogenesis, and lymphangiogenesis.
SIX1 was recently shown to have pro-oncogenic and metastatic properties in different tumors (Coletta et al., 2008; Wang et al., 2012). Interestingly, to the best of our knowledge, there are no studies available in melanoma. In addition, SIX1 is capable of inducing lymphangiogenesis by increasing VEGFC expression (Liu et al., 2014; Wang et al., 2012). The high expression of Angpt2, Six1, and Vegfc in 4C11+ cells suggests that they might contribute together to the vascular phenotype of these cells.

Then, we blocked the VEGF pathway in 4C11+ cells using axitinib, a potent and selective inhibitor of VEGFRs (Zhang et al., 2014). This drug competitively binds to the intracellular ATP site domain of the receptors, stabilizing them in an inactive conformation and therefore inhibiting downstream signal transduction (Gross-Goupil et al., 2013). The treatment induced a significant G2/M arrest, reduced 4C11+ cells ability to migrate in vitro and to develop tumors in vivo. As axitinib is a classical inhibitor of angiogenesis, most studies analyzed the effects of the drug in an already developed tumor (He et al., 2014; Zhang et al., 2014), but we aimed to examine the ability of cells previously treated with the drug to develop a tumor. While 4C11+ control cells developed big tumor masses, axitinib-pretreated 4C11+ cells only gave rise to small, noninvasive tumors. This is presumably due to the inhibition of VEGFC signaling—the only VEGF ligand expressed in these cells—which leads to an impaired tumor development. Indeed, axitinib has been reported to have antitumor activity in highly angiogenic tumors (Fruehauf et al., 2011).

Axitinib has previously been shown to induce senescence in gastric cancer (He et al., 2014) and glioma cells (Morelli et al., 2016), a phenotype suggested by the G2/M arrest observed in 4C11+ -treated cells. Although senescence can influence tumor growth (Rodier and Campisi, 2011), former studies have shown that major axitinib effects are due to the VEGF pathway blockade. Nonetheless, the profound abrogation of tumor growth caused by the VEGF receptors blockade, and consequent inhibition of VEGFC function, suggests the VEGFC pathway has an important role in the aggressive phenotype of 4C11+ cells. Further studies of our group should elucidate whether lymphangiogenesis is involved in 4C11+ tumor cells dissemination.

To evaluate the translational relevance of our findings, we performed a Cox multivariate analysis for the expression of VEGFC, ANGPT2, MET, SIX1, and VEGFR-3. After adjustment for the covariates, high expression of VEGFR-3 and ANGPT2 were both independent predictors of poor prognosis. Kaplan–Meier analysis illustrated that patients with low and high expression of these genes have distinct survival curves, although log-rank tests were not significant. Reinforcing these data, IHC and MELC staining demonstrated that tissues obtained from metastatic melanoma patients, which are known to have a worse prognosis, have higher expression levels of VEGFR-3 and ANGPT2 compared with tissues obtained from patients with primary melanoma. In the samples analyzed by the MELC technique, VEGFR-3 was mainly detected in the tumor vasculature, while ANGPT2 was highly expressed by the tumor cells. Although these molecules are predominantly expressed by endothelial cells, recent studies have reported their expression by tumor cells (Streit and Detmar, 2003; Su et al., 2008a, 2008b). Notably, VEGFR-3 and ANGPT2 were previously observed to be expressed in melanoma tumor cells (Helfrich et al., 2009; Mouawad et al., 2009).

Vascular endothelial growth factor receptor-3 expression was already shown to be a prognostic marker of disease-free survival in gastric adenocarcinoma (Jüttner et al., 2006), but there are only a few studies in melanoma. Soluble VEGFR-3 has been reported to be associated with disease-free survival but not overall survival in melanoma patients (Mouawad et al., 2009). Moreover, ANGPT2 expression was correlated with overall patient survival in colorectal and breast cancer (Hong et al., 2017; Sfiligoi et al., 2003). In melanoma, one study demonstrated high levels of circulating ANGPT2 to be associated with poor patient overall survival (Helfrich et al., 2009). To our knowledge, we are the first to show that ANGPT2 (HR = 1.189; P = 0.002) and VEGFR-3 (HR = 1.999; P = 0.044) are independent predictors of prognosis in melanoma patients.

Furthermore, we detected that Vegfc, Angpt2, and Six1 promoters were hypomethylated in 4C11+ cells compared with 4C11− cells. Importantly, the methylation status of these genes was inversely correlated to their mRNA expression levels. Indeed, treatment of 4C11− cells with 5-Aza-CdR increased the expression of the three genes, suggesting that DNA methylation regulates their transcription. Interestingly, we found low levels of VEGFC, ANGPT2, and SIX1 promoter methylation are independent prognostic factors of poor patient survival. VEGFC expression has previously been shown to predict melanoma patient survival (Boone et al., 2008; Liu et al., 2008), and its expression has been shown to be regulated by DNA methylation in gastric cancer (Matsumura et al., 2007). Consistently, VEGFC promoter methylation was reported to be associated with progression-free survival.
in ovarian cancer (Dai et al., 2013). Concerning ANGPT2, the methylation status of 6 CpGs near the gene transcription site (four of which were analyzed in this study) has already been shown to predict overall survival of chronic lymphocytic leukemia patients (Martinelli et al., 2013). Several studies have reported that SIX1 overexpression is frequently associated with poor patient prognosis in various malignancies, as colo-rectal cancer (Kahlert et al., 2015) and glioma (Zhang and Xu, 2017), however not in melanoma. Besides, the mechanisms responsible for the high expression of SIX1 have been poorly investigated. Methylation of SIX1 promoter has previously been reported as a transcription regulatory mechanism in the porcine and bovine muscle (Wei et al., 2018; Wu et al., 2013). To our knowledge, this is the first report to show VEGFC (HR = 0.035; \( P = 0.031 \)), ANGPT2 (HR = 0.168; \( P < 0.001 \)), and SIX1 (HR = 0.591; \( P = 0.009 \)) promoter methylation as prognostic markers for melanoma patient survival.

5. Conclusion

In summary, we found that the VEGFC pathway is highly correlated with tumor aggressiveness in our murine model. Moreover, we identified VEGFR-3 and ANGPT2 expression, as well as VEGFC, ANGPT2, and SIX1 promoter methylation, as independent prognostic factors for overall survival in melanoma patients, showing the high translational relevance of our findings obtained in a murine model.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

ACM, JKM, RSS, and MGJ conceived and designed the study, interpreted the data, and participated in manuscript preparation. ACM performed most experiments and drafted the manuscript. ACM and JKM performed the CAM assays. FER carried out the methylome analysis. FA and AF performed the survival analysis. CO performed the IHC and MELC analysis on human specimens. CIG digitalized the CAM tissue sections for CaseViewer evaluation. AH and AG performed the CAM histological examinations. All authors read and approved the final manuscript.

Authors’ information

The present work was performed in partial fulfillment of the requirements for obtaining the PhD degree ‘Dr.rer. nat.’ at the FAU Erlangen-Nürnberg for ACM. ACM was a Cotutelle student at FAU and UNIFESP being integrated in the IZKF doctoral program of the FAU.

Data availability

The TCGA Skin Cutaneous Melanoma project data can be found in https://portal.gdc.cancer.gov/projects/TCGA-SKCM.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Representative macroscopic images and HE stained tissue sections (4× magnification) of 4C11+ tumors grown on the CAM for 5 days.

**Fig. S2.** Representative macroscopic image and HE staining (4× and 40× magnification) of tumors grown on the CAM. 4C11+ cells were pretreated *in vitro* with 1 µM of Axitinib for 48 h and applied onto the CAM. Tumors grown were removed after 5 days.

**Fig. S3.** Representative images of VEGFR-3 and ANGPT2 staining in normal skin and colon, which were used as negative controls of the IHC staining.

**Table S1.** Primers sequences for RT-qPCR reactions.

**Table S2.** mRNA upregulated in 4C11+ cells in comparison to 4C11− cells as assessed by the NanoString Panel.

**Table S3.** mRNA downregulated in 4C11+ cells in comparison to 4C11− cells as assessed by the NanoString Panel.

**Table S4.** CpGs differentially methylated of *Vegfc*, *Angpt2* and *Six1* promoters regions in 4C11+ cells compared to 4C11− cells evaluated by ERRBS.