A novel function of Junctional Adhesion Molecule-C in mediating melanoma cell metastasis

Harald F. Langer¹,²,*, Valeria V. Orlova¹,³,*, Changping Xie⁴,*, Sunil Kaul¹,*, Darius Schneider⁴, Anke S. Lonsdorf⁵,⁶, Manuela Fahrleitner², Eun Young Choi¹,§, Vanessa Dutoit⁴, Manuela Pellegrini⁷, Sylvia Grossklaus⁸,⁹, Peter P. Nawroth⁴, Gustavo Baretton¹⁰, Sentot Santoso¹¹, Sam T. Hwang⁵,¹², Bernd Arnold¹³, and Triantafyllos Chavakis¹,⁴,⁸,⁹

¹Experimental Immunology Branch, National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD 20892, USA; ²Medizinische Klinik III, Kardiologie und Kreislaufkrankungen, University Tübingen, 72076 Tübingen, Germany; ³Molecular Cell Biology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands; ⁴Department of Medicine I, University Heidelberg, 69120 Heidelberg, Germany; ⁵Dermatology Branch, NCI, NIH, Bethesda, MD 20892, USA; ⁶Department of Dermatology, University Heidelberg, 69120 Heidelberg, Germany; ⁷Department of Public Health and Cellular Biology, University of Rome ‘Tor Vergata’, 00133 Rome, Italy; ⁸Division of Vascular Inflammation, Diabetes and Kidney, Department of Medicine, ⁹Institute of Physiology and ¹⁰Institute for Pathology, University Dresden, 01307 Dresden, Germany; ¹¹Institute for Clinical Immunology and Transfusion Medicine, 35390 Giessen, Germany; ¹²Department of Dermatology, Medical College of Wisconsin, Milwaukee, WI 53226, USA; ¹³Molecular Immunology, German Cancer Research Center, 69120 Heidelberg, Germany

*, contributed equally
§, current address: Graduate School of Medicine, University of Ulsan, Seoul 138-736, Korea

Short title: JAM-C in melanoma metastasis

Correspondence to: Dr. T. Chavakis (triantafyllos.chavakis@uniklinikum-dresden.de)
Abstract

Hematogenous dissemination of melanoma is a life-threatening complication of this malignant tumor. Here, we identified Junctional Adhesion Molecule-C (JAM-C) as a novel player in melanoma metastasis to the lung. JAM-C expression was identified in human and murine melanoma cell lines, in human malignant melanoma, as well as in metastatic melanoma including melanoma lung metastasis. JAM-C expressed on both murine B16 melanoma cells as well as on endothelial cells, promoted the transendothelial migration of the melanoma cells. We generated mice with inactivation of JAM-C. JAM-C-/- mice as well as endothelial-specific JAM-C-deficient mice displayed significantly decreased B16 melanoma cell metastasis to the lung, whereas treatment of mice with soluble JAM-C prevented melanoma lung metastasis. Together, JAM-C represents a novel therapeutic target for melanoma metastasis.
Introduction

Malignant melanoma has high metastatic potential. Blood-borne melanoma metastasis to distant organs including the lung is associated with high mortality (1, 2). Blood-borne metastasis requires the invasion of the tumor into blood vessels and the subsequent extravasation; the latter process is mediated by a multitude of tightly coordinated adhesive interactions between the tumor cells and the endothelium of the venules, capillaries and postcapillary venules of the target organ (1, 3). In analogy to the leukocyte adhesion cascade, tumor cell firm arrest to the endothelium is mediated at least in parts by adhesion receptors of the integrin and immunoglobulin family. For instance, the integrin VLA-4 (\(\alpha 4\beta 1\)) on melanoma cells mediate adhesion on endothelial VCAM-1 thereby promoting extravasation of intravenously injected tumor cells and their metastasis to the lung (4-7). Furthermore, endothelial Thy-1 may mediate \(\alpha v\beta 3\)-dependent melanoma cell adhesion (8), whereas melanoma cell adhesion molecule is also associated with the metastatic phenotype of melanoma cells (9). These adhesive interactions can be triggered by chemokine receptors and their ligands, such as CXCR4 and CXCL12 (SDF-1alpha) (4). However, it is conceivable that further adhesion receptors that have been implicated in the leukocyte adhesion cascade as well as to regulate the endothelial barrier may participate in the process of melanoma cell adhesion.

Junctional adhesion molecule (JAM)-C is the third member of the JAM family that consists of two Ig-like domains and has a PDZ domain-binding motif at its carboxy-terminal region (10-12). JAM-C is expressed in endothelial and some epithelial cells, partially localizing to tight junctions, as well as on platelets and some lymphocyte subsets (13-15). JAM-C has been implicated in leukocyte recruitment (16-18) through its propensities to undergo homophilic binding, heterophilic interactions with the leukocyte integrin Mac-1 or JAM-B (19, 20) or to disrupt the
endothelial barrier by counteracting the activity of the small GTPase Rap1a, thereby inhibiting VE-Cadherin-mediated cell-cell junctions and integrin function (14, 21). While JAM-C expression has recently been identified on mouse melanoma cells (22), its participation in the metastatic process of melanoma has not been studied yet. These observations prompted us to investigate the function of JAM-C in melanoma metastasis. We identified JAM-C expression in human primary and metastatic melanoma and showed that JAM-C mediated transmigration of melanoma cells through endothelial cells, whereas JAM-C blockade in vivo prevented lung metastasis in a murine B16 melanoma model. Moreover, we generated mice with conditional deletion of JAM-C and found that complete or endothelial-specific JAM-C deletion decreased hematogenous melanoma metastasis to the lung. Thus, JAM-C may represent a novel therapeutic target in melanoma metastasis.
Materials and Methods

The following Methods related to supplementary data are included in the supplementary online materials: “Reagents” “Isolation of total RNA and real time PCR analysis”, “Generation of JAM-C conditional knockout mice”, “Western blot analysis”, and “Immunohistochemistry of human lungs”

Cell Culture and transfection

B16 melanoma cells retrovirally transduced with cDNA encoding firefly (*Photinus purpureus*) luciferase were grown in DMEM (Invitrogen) with 10% FBS and supplements, including puromycin (4). In some experiments B16 cells additionally transduced with cDNA encoding CXCR4 or the empty retroviral vector pLNCX2 alone were used. These cells were cultured in DMEM with 10% FBS and supplements, including puromycin and G418 (4). Moreover, the human melanoma cell lines SkMel28, IGR1, MeWo and C8161 were used. B16 cells were originally from the cell line repository at NCI Frederick and were maintained at the lab of Dr. Sam Hwang (NCI, NIH), from whom cells were obtained, whereas human cell lines were provided by Dr. A. Lonsdorf (Dermatology, University Heidelberg). B16 and SkMel28 cells were verified by molecular profiling at the respective providing labs. MeWo and IGR1 cells were originally from Cell-Lines Service (Eppelheim, Germany) and C8161 cells were from Lonza (Cologne, Germany). IGR1, MeWo and C8161 cells used derived from the original purchased stocks. Mouse endothelioma b.End.3 cells were from American Type Culture Collection (ATCC; Manassas, VA). Human dermal microvascular endothelial cells were previously described (14).

For transfection of B16 or b.End.3 cells with siRNA, the following chemically synthesized duplex siRNAs were engaged: siRNA directed against mouse JAM-C (giGENOME ON-TARGETplus mouse_JAM-C smartpool L-044688 and ON-
TARGETplus Set of 4 Upgrade: LU-044688, including 4 different siRNAs against JAM-C, siRNA-09, siRNA-10, siRNA-11 and siRNA-12) or a respective control non-targeting siRNA (giGENOME ON-TARGETplus Non-Targeting Pool D-001810) (both from Dharmacon). siRNAs were transfected as described (14).

CHO cells were cultivated as described (20). The motif RIE at positions 64-66 mediating the homophilic binding of JAM-C (20) is conserved amongst species. In order to generate mutant mouse JAM-C, we engaged the original vector pcDNA3-mJAMC (provided by Dr. Tanja Keiper, University Heidelberg, Germany) and QuikChange mutagenesis (Stratagene) was performed. The following mismatches were introduced: GA->GC and GA->CG at positions 196 and 197 resulting in the mutations E66A and E66R, respectively. Constructs were validated by nucleotide sequencing analysis. The expression vector with WT or mutant JAM-C or control vector plasmid pcDNA3-Zeo were transfected into CHO cells using Lipofectamine Plus reagent (Invitrogen). Expression was monitored by western blot.

**Adhesion assays**

To evaluate B16 melanoma cell adhesion, 96-well plates were coated with JAM-C-Fc (10 µg/ml), VCAM-1-Fc (10 µg/ml) or fibronectin (10 µg/ml) overnight and blocked with BSA (3%). Subsequently, melanoma cells (5x10⁴/well) were added and incubated for 40 min. After two gentle washing steps, adherent B16 cells were counted by direct phase contrast microscopy. In other experiments, b.End.3 cells transfected with different siRNAs, or non-transfected CHO cells or CHO cells transfected with vector control, JAM-C, JAM-C-RIA mutant or JAM-C-RIR mutant were grown in 96-well plates. Plates were blocked with 3% BSA for 30 min at 22°C. Subsequently, B16 cells (5x10⁴/well) were added to confluent b.End.3 or CHO cells for 40 min at 37°C. After washings, adherent cells were quantified (23). Adhesion of
SkMel28 cells to human dermal microvascular endothelial cells was studied as described (20).

Adhesion experiments under flow conditions were performed as described (23). Briefly, glass coverslips were coated with BSA, mouse JAM-C-Fc (10 µg/ml), mouse VCAM-1-Fc (10 µg/ml) and used in a flow chamber (Oligene, Berlin, Germany). Where indicated, melanoma cells were pretreated with JAM-C-Fc (10 µg/ml) for 1 h before perfusion. Perfusion of B16 cells (2.5x10^5/ml) was performed at shear rates of 500 s\(^{-1}\) or 1000 s\(^{-1}\). Experiments were recorded in real time on video-CD and evaluated off-line by counting the number of adherent cells in 5 different fields after 10 min of perfusion.

**Isolation of primary mouse endothelial cells and FACS analysis**

Primary mouse lung endothelial cells were isolated from lungs of VEC-Cre\(^{-}\):JAM-C\(^{\text{flox/}}\), VEC-Cre\(^{-}\):JAM-C\(^{\text{flox/}}\) and VEC-Cre\(^{+}\):JAM-C\(^{\text{flox/}}\) mice as well as from lungs of SCL-Cre-ERT\(^{-}\):JAM-C\(^{\text{flox/flox}}\) and SCL-Cre-ERT\(^{+}\):JAM-C\(^{\text{flox/flox}}\) mice as described (24, 25). For tamoxifen-inducible deletion of JAM-C in SCL-Cre-ERT\(^{+}\):JAM-C\(^{\text{flox/flox}}\) endothelial cells ex vivo, cells were incubated in media containing 4-OHT (1µM) for 72h. Endothelial cells were characterized by FACS staining with APC conjugated anti-PECAM-1 (clone 390) (eBioscience) or FITC conjugated antibody to VE-Cadherin (clone BV13) (eBioscience). Endothelial cell-specific deletion of JAM-C was assessed by double FACS staining with FITC conjugated anti JAM-C (clone CRAM F26) (Abcam) and APC conjugated anti-PECAM-1 antibody.

**Proliferation assay**

B16 cell proliferation was performed as described (25, 26).
Transmigration assay

Transmigration of B16 cells was performed using 6.5 mm transwells with 8 µm pore size (Costar). Mouse b.End.3 cells or primary endothelial cells isolated from mouse lungs were seeded on transwell filters 2 days prior to the assay and grown to confluency in the upper compartment at 37°C, 5% CO₂. At the beginning of the transmigration assay 600 µl medium containing SDF-1 (200 ng/ml, serum free DMEM, 0.3% BSA) was added to the lower compartment of the transwell system. B16 cells (2x10⁵/well) were added to the upper compartment on top of the endothelial monolayer in a total volume of 100 µl. After incubation for 24 h at 37°C, cells from the upper chamber were removed with a cotton swab and the filters were removed, fixed with ice cold methanol for 20 min and stained with crystal violet for 30 min. After washing of the filters in distilled water, they were mounted on glass cover slips and the number of transmigrated B16 cells was quantified on the lower side of the filter by counting under a microscope.

In vivo lung metastasis

Experiments were approved by the NCI Animal Care and Use Committee. Luciferase-expressing B16 cells in the exponential growth phase were harvested by trypsinization and washed twice before injection. Cell viability was >95% as determined by trypan blue dye exclusion. 4x10⁵ B16 cells in 200 µl PBS were injected into the tail vein of mice. JAM-C⁺⁺ or littermate JAM-C⁺⁺ mice or endothelial-specific JAM-C-sufficient and JAM-C-deficient mice were engaged. For studies with inhibitors we used C57BL/6 mice. In these experiments soluble mouse JAM-C (smJAM-C) expressed with a 6His tag (27) or control 6His-peptide (each at 100 µg/mouse) in sterile PBS were administered via an i.p. route at day 0, 3, 7, and 10 after tumor cell inoculation. Luciferase activity in the lung due to B16 cells metastasizing to the lung
was measured using a luciferase reporter assay system (Promega) on days 14 or 21, as described (28). For tissue analysis, lungs from each animal were homogenized in 1 ml of Glo Lysis buffer (Promega), of which 100-µl aliquots were then assayed in duplicates. Means of duplicates were used to represent the luciferase activity for a given tissue from a particular animal. Lysates were assayed in the presence of luciferin (1:1) using a Thermo Labsystems MLX 96-well luminometer (Helsinki, Finland). Units are relative light units (RLU).

In some experiments, at indicated time points, 150 µl of D-luciferin (25 mg/ml) was injected i.p. into mice. Mice were then anaesthetized with isoflurane and luciferase activity was visualized for 1–3 min in a pitch-black chamber with a cooled CCD camera (Xenogen, Alameda, CA), principally as described before (28). Images were analyzed with the LivingImage software package system (Xenogen). Furthermore, in some of the experiments mice were anaesthetized with isoflurane and analyzed in a small animal CT scan (Siemens).

In other experiments, $10^5$ non-transfected B16 cells were injected i.v. and mice were treated on days 0, 1, 2, 4, 7 and 10 with an intraperitoneal injection of smJAM-C or control 6His-peptide (each at 3mg/kg body weight), and 21 days after induction, mice were sacrificed, their lungs were excised, fixed in 10% formalin and embedded in paraffin. Tissue blocks were cut into 8µm sections and subsequently stained with haematoxylin and eosin and slides were analyzed microscopically.

**Immunohistochemistry**

A tissue microarray kit (ME1001; US Biomax Inc, Rockville, MD) was used. This kit contained samples from 56 patients with malignant melanomas, 20 patients with metastatic malignant melanomas and 24 patients with benign nevus. Samples were processed for immunohistochemistry according to the manufacturer’s instructions.
Metastatic malignant melanoma samples were from lymph node metastasis, whereas non metastatic malignant melanoma samples were from different locations of the skin including the right buttocks, back, left foot, sole, left upper arm, right heel, right thigh, waist, left heel, left shoulder and the thumb. Paraffin-embedded specimen was deparaffinized in xylene and rehydrated through graded ethanol washes (100% to 70%). Endogenous peroxidase activity was blocked using H₂O₂. Antigen retrieval was performed using 1 mM EDTA (pH 8.0). Slides were preincubated with 5% normal goat serum, then incubated with anti-JAM-C (30 μg/ml), followed by a biotinylated secondary antibody. For color reaction, a DAB substrate kit (Vectastain ABC systems, Vector laboratories) was used. Sections were counterstained with hematoxylin. Slides were covered with mounting medium (Southern Biotechnology) and analyzed. Color intensity was evaluated using MetaMorph software (v6.1) (Molecular Devices, PA) and is presented as color intensity per area.

**Data presentation and statistics**

Comparisons between group means were performed using Student t-test analysis or ANOVA as appropriate. P<0.05 was considered as statistically significant. Data are presented as mean+SEM.
Results

Expression of JAM-C in human melanoma and melanoma metastasis.

Recently, JAM-C was found to be expressed on B16 mouse melanoma cells (22). We verified the expression of JAM-C in mouse B16 cells by western blot. Moreover, we tested the expression of JAM-C in different human melanoma cell lines. In particular, SkMel28 and IGR1 cells expressed JAM-C, whereas the cell lines MeWo and C8161 expressed little or no JAM-C, respectively (supplementary figure 1A and data not shown). To address the potential clinical relevance of JAM-C expression in human melanoma, we assessed its expression by immunohistochemistry in a human melanoma tissue array. This array included samples from patients with benign nevi, malignant melanomas or metastatic malignant melanomas. This analysis revealed that JAM-C was expressed in benign nevi and in malignant melanomas. Interestingly, JAM-C expression was significantly enhanced in metastatic malignant melanoma (Fig. 1A and B). Furthermore, we performed immunohistochemistry for JAM-C in sections from 5 different lung metastases derived from lung excisions. All 5 melanoma metastasis lung specimens stained positive for JAM-C (supplementary figure 1B and data not shown).

JAM-C mediates B16 melanoma cell transendothelial migration.

Recently, JAM-C expressed on a fibrosarcoma cell line was shown to promote adhesion of these tumor cells to extracellular matrix (ECM) proteins (22,29). Thus, we tested the hypothesis that JAM-C may functionally contribute to melanoma metastasis. To analyze the potential role of JAM-C on B16 cells for potential interactions with the vascular wall we tested adhesion of these cells under different shear conditions. B16 cells adhered to immobilized JAM-C predominantly under low shear rates (500 s\(^{-1}\)), whereas adhesion of the tumor cells to VCAM-1 was found
under both high and low shear rates (500 s\(^{-1}\) and 1000 s\(^{-1}\)) (Fig. 2A). VCAM-1 is recognized by the integrin VLA-4 on B16 cells and this interaction has been implicated in melanoma metastasis (4, 6, 7). Interestingly, at low shear rates, the adhesion of B16 cells to JAM-C reached virtually the level of adhesion to immobilized VCAM-1 (Fig. 2A). Low shear rates are relevant for melanoma metastasis, which to a large extent takes place in the capillary bed (30).

Preincubation of B16 cells with smJAM-C significantly inhibited their adhesion to immobilized JAM-C (Fig. 2B), whereas beta1-integrin-dependent (4-7) adhesion to VCAM-1 was not affected by smJAM-C (Fig. 2C). These data suggest that the homophilic interaction of JAM-C may mediate arrest of B16 cells onto immobilized JAM-C. To provide further evidence for this hypothesis we performed siRNA-mediated knockdown of JAM-C in B16 cells. Efficient JAM-C knockdown was monitored by western blot (supplementary figure 2A). B16 cell adhesion to JAM-C was significantly decreased by siRNA-mediated knockdown of JAM-C (Fig. 2D). In contrast, JAM-C knockdown did not affect the beta1-integrin-dependent B16 cell adhesion to VCAM-1 or fibronectin (Fig. 2D), suggesting that JAM-C levels did not regulate beta1-integrin activity in B16 cells.

To further assess the homophilic binding of JAM-C in adhesive interactions of B16 cells, we studied B16 cell adhesion to CHO cells expressing different JAM-C isoforms (Fig. 2E and supplementary figure 3). Expression of JAM-C in CHO cells promoted a four-fold increase in B16 cell adhesion as compared to non-transfected or vector-transfected CHO cells. JAM-C dimerization is mediated by the sequence Arg64-Ile65-Glu66 of JAM-C (20). CHO cells expressing JAM-C isoforms mutated at Glu66 to either Arg66 or Ala66 (RIE motif at 64-66 mutated to RIR or RIA, respectively) resulting in disruption of the putative salt bridge and the homophilic interaction of JAM-C failed to promote adhesion of B16 cells above background (non-
transfected or control-transfected cells) (Fig. 2E). Thus, the homophilic binding of JAM-C is engaged in adhesive interactions of B16 melanoma cells.

We next tested the importance of JAM-C-mediated adhesive interactions for melanoma cell adhesion to endothelial cells and transendothelial migration, as both adhesion and transmigration of circulating tumor cells through the endothelium are prerequisites for hematogenous metastasis. Knockdown of JAM-C by siRNA in either B16 cells or mouse endothelial cells did not affect endothelial adhesion of melanoma cells (supplementary figure 4A and 4B). Moreover, adhesion of human SkMel28 cells to human dermal endothelial cells did not depend on JAM-C (supplementary figure 4C).

We then evaluated the role of JAM-C for transendothelial migration of B16 cells. Transmigration of B16 melanoma cells across mouse endothelial cells was significantly reduced by siRNA-mediated knockdown of JAM-C in either the B16 cells or the endothelial cells (Fig. 3A and 3B). To ascertain specificity of siRNA-mediated knockdown, we tested 4 different siRNAs against JAM-C. Western blot analysis for JAM-C protein upon transfection with control-siRNA or different JAM-C-targeting siRNAs is shown in supplementary figure 2A. Knockdown of JAM-C in B16 cells by different siRNAs resulted in significant reduction of their transendothelial migration (supplementary figure 2B). Furthermore, soluble mouse JAM-C expressed as an Fc fusion protein (JAM-C-Fc) but not control Fc protein significantly decreased B16 transendothelial migration (supplementary figure 4D). In contrast, proliferation of B16 melanoma cells was not affected by JAM-C knockdown (data not shown).

Moreover, we analyzed the expression of molecules involved in melanoma cell migration or invasion upon knockdown of JAM-C. We found no difference in the expression of adhesion molecules, such as integrin beta1 or integrin alpha4 that have been implicated in the adhesion of melanoma cells to the endothelium (4-7).
(supplementary figure 5). In addition, JAM-C knockdown did not affect the expression of molecules involved in invasiveness, such as MMP-9 or MT1-MMP.

**Generation of JAM-C conditional mice**

In order to address the effects of JAM-C deficiency in more detail, we generated mice with conditional inactivation of JAM-C (JAM-C floxed). Exon 1 of JAM-C was flanked by two loxp sites, whereas the neomycin resistance gene was flanked by flt sites (supplementary figure 6). Deletion of the neomycin resistance gene by crossing with Flp deleter mice resulted in a JAM-C floxed allele. A homozygous JAM-C floxed colony was generated (JAM-C^floxfloxfloxflox). Embryonic deletion of JAM-C was performed utilizing EIIa-Cre transgenic mice, resulting in mice with a JAM-C null allele. By crossing JAM-C^+/- mice, we obtained JAM-C^-/- mice with a frequency at birth slightly lower that the Mendelian ratio (17%). In accordance with previously published complete JAM-C-deficient mice (31,32), we observed pre-wean mortality of null mice resulting in an almost three-fold reduction of JAM-C^-/- mice surviving to adulthood (6% at weaning vs. 17% at birth). In addition, JAM-C-deficient males were infertile (33). The absence of JAM-C mRNA and protein from complete knockout mice was verified, whereas JAM-C^floxfloxfloxflox mice displayed normal levels of JAM-C mRNA and protein (Fig. 4A and supplementary figure 6B) and did not show any male infertility. These data indicate that JAM-C^floxfloxfloxflox mice were appropriate for conditional JAM-C deletion.

JAM-C^floxfloxfloxflox mice were crossed with mice that were transgenic for the tamoxifen-inducible Cre-ERT recombinase driven by the 5' endothelial enhancer of the stem cell leukemia (SCL) locus (endothelial-SCL-Cre-ERT) (34). We isolated primary endothelial cells from the lungs of SCL-Cre-ERT^+:JAM-C^floxfloxflox and of SCL-Cre-ERT^-:JAM-C^floxfloxflox mice (24, 25). Addition of 4-OHT (4-hydroxytamoxifen) to
these cells resulted in efficient \textit{ex vivo} deletion of JAM-C in SCL-Cre-ERT\textsuperscript{+}:JAM-C\textsuperscript{flox/flox} cells, but not in SCL-Cre-ERT\textsuperscript{-}:JAM-C\textsuperscript{flox/flox} endothelial cells (Fig. 4B). SCL-Cre-ERT\textsuperscript{+}:JAM-C\textsuperscript{flox/flox} endothelial cells that were treated without or with 4-OHT (i.e. JAM-C-sufficient and JAM-C-deficient cells) were studied in \textit{in vitro} transmigration assays. Genomic deletion of JAM-C in the SCL-Cre-ERT\textsuperscript{+}:JAM-C\textsuperscript{flox/flox} cells by 4-OHT resulted in significantly reduced B16 cell transmigration (Fig. 4C).

**JAM-C is crucial for melanoma lung metastasis \textit{in vivo}**.

To evaluate the function of JAM-C in melanoma metastasis \textit{in vivo}, we used a lung metastasis model (4). JAM-C\textsuperscript{+/+} or JAM-C\textsuperscript{-/-} mice were injected via the tail vein with B16 cells bearing luciferase activity and after 3 weeks mice were sacrificed and luciferase activity was quantified in lung lysates. Compared to JAM-C-sufficient mice, JAM-C\textsuperscript{-/-} mice displayed significantly reduced lung metastasis. Metastasis was reduced in JAM-C-deficient mice that were backcrossed for 7 generations to the BL/6 background as compared to their littermate WT mice (Fig. 5A), and similar findings were observed with littermate JAM-C-sufficient and JAM-C-deficient mice that were in a mixed 129:B6 background (supplementary figure 7). These findings were corroborated by performing small animal computer tomography (CT) scan of the lungs of JAM-C\textsuperscript{+/+} and JAM-C\textsuperscript{-/-} mice and \textit{in vivo} bioluminescence imaging. Both approaches revealed decreased melanoma metastasis in the lungs of JAM-C-deficient mice (Fig. 5B and supplementary figure 8).

To verify that JAM-C expressed on endothelial cells was important for melanoma lung metastasis, we generated endothelial-specific JAM-C-deficient mice. Constitutive endothelial-specific deletion of JAM-C was performed by generating mice heterozygous for the floxed and deleted JAM-C allele (JAM-C\textsuperscript{flox/-}), which carried concomitantly the Cre-recombinase under the control of the VE-cadherin-promoter
(VEC-Cre⁺:JAM-Cfloxl−) (35). Real-time PCR analysis of lung endothelial cells isolated from the VEC-Cre⁺:JAM-Cfloxl− mice demonstrated efficient deletion of the floxed JAM-C allele, as compared to control mice (VEC-Cre−:JAM-Cfloxl+ and VEC-Cre−:JAM-Cfloxl− mice) (Fig. 6A). Endothelial-specific JAM-C deletion was associated with a significant decrease in B16 melanoma lung metastasis (Fig. 6B). In other words, endothelial JAM-C promotes melanoma lung metastasis.

Since JAM-C−/− and endothelial-specific JAM-C-deficient mice showed reduced melanoma lung metastasis, we tested whether targeting of JAM-C in a prophylactic therapeutic setting results in reduced metastatic B16 melanoma tumor burden in the lung. To do so, C57BL/6 mice were treated with smJAM-C or control protein at different days after i.v. tumor cell injection. Strikingly, treatment with smJAM-C resulted in dramatic reduction of B16 cell metastasis to the lung at 2 weeks after tumor cell instillation, as compared to mice receiving control protein (Fig. 6C). In addition, immunohistology analysis of the lungs from mice receiving smJAM-C treatment or from the control group was performed. Whereas lungs of mice from the control group displayed numerous and large metastases, the overall degree of metastasis was decreased (less and smaller metastases) in the smJAM-C treatment group (supplementary figure 9).
Discussion

In the present study we demonstrated for the first time the role of JAM-C in promoting hematogenous melanoma lung metastasis. JAM-C was expressed in human melanoma as well as in metastatic melanoma and in lung metastasis. Moreover, JAM-C was expressed on human melanoma cell lines and in murine B16 melanoma cells. JAM-C-dependent adhesive interactions promoted the transendothelial migration of melanoma cells \textit{in vitro}. \textit{In vivo}, complete or endothelial-specific deletion of JAM-C resulted in significantly decreased melanoma lung metastasis, whereas JAM-C blockade also dramatically reduced hematogenous melanoma spreading to the lung. Thus, JAM-C may represent a novel therapeutic target for melanoma metastasis, and anti-JAM-C therapies may have clinical applications for prevention of metastatic disease.

JAM-C on both melanoma and endothelial cells mediated the transendothelial migration of B16 cells, as JAM-C knockdown in either cell reduced transendothelial migration of melanoma cells. Moreover, soluble JAM-C, which blocks the homophilic interaction of JAM-C, reduced B16 transendothelial migration. In contrast, JAM-C knockdown in either B16 cells or the endothelium did not affect melanoma cell adhesion to the endothelium. In addition, JAM-C knockdown did not affect the beta1-integrin-dependent B16 cell adhesion to VCAM-1 or fibronectin, suggesting that JAM-C levels did not regulate beta1-integrin activity in B16 cells. This observation is somewhat different from the JAM-C-mediated regulation of integrin-dependent adhesion in other cells, such as endothelial cells (21,29). Together, our experiments suggest that JAM-C, via its homophilic interaction, is operative in mediating melanoma cell transendothelial migration. In other words, transendothelial migration of B16 cells was mediated by the interaction of JAM-C on the B16 cell surface with JAM-C in endothelial junctions. In contrast, JAM-C does not participate in melanoma
cell adhesion to the endothelium. This could be explained by the fact that the majority of JAM-C in endothelial cells is not present on the apical surface but is predominantly found in tight junctions (11,14). Thus, the localisation of JAM-C in endothelial junctions determines that the homophilic interaction between melanoma JAM-C and endothelial JAM-C mediates melanoma cell transendothelial migration.

Noteworthy, human melanoma cell lines SkMel28 and IGR1 expressed JAM-C, whereas MeWo and C8161 cell lines expressed little or no JAM-C. This probably reflects the fact that melanomas and cell lines resulting from melanomas are heterogeneous and have a high degree of plasticity. In addition, epigenetic changes due to the influence of the tumor microenvironment could severely affect the tumor cell phenotype (36).

Herein, we also report for the first time the generation of mice with conditional JAM-C inactivation. Complete JAM-C deletion yielded mice with a high degree of pre-wean mortality and male infertility (31,32). However, JAM-C floxed mice bypass these problems and could be engaged as a helpful tool to study the cell-specific function of this molecule in physiology and pathology.

A molecule with structural homology to JAM-C, the endothelial-cell selective adhesion molecule, which has been demonstrated to function similar to JAM-C in promoting endothelial barrier disruption (37), was recently shown to promote melanoma lung metastasis (38), in parts by modulating the vascular density of the metastatic tissues. It would be worth to address whether JAM-C could also regulate vascularisation of metastatic tissues in the future. Furthermore, in a recent study JAM-C promoted glioma growth and invasion via its interaction with JAM-B (39). It would be interesting to address in future studies whether the JAM-C/JAM-B crosstalk also participates in melanoma metastasis.
Interestingly, smJAM-C dramatically inhibited pulmonary metastasis to an extent that was higher than JAM-C deficiency. This is probably due to the fact that smJAM-C may act on both melanoma and endothelial cells, thus, providing a stronger inhibitory effect. Together, our data unequivocally suggest that JAM-C blockade may be a promising strategy as an adjuvant therapy or preventive therapy for melanoma metastasis.

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References

Figure legends

**Fig. 1: JAM-C is expressed in human melanoma**

(A,B) Samples of patients with benign nevi (n=24), malignant melanomas (n=56) or malignant metastatic melanomas (n=24) on a tissue microarray were analyzed by immunohistochemistry using a mAb to human JAM-C. (A) Representative images are shown in 10x (upper panel) or 40x magnification (lower panel). (B) The color intensity per area was analyzed using Metamorph software. *, p<0.05

**Fig. 2: B16 melanoma cells engage JAM-C for adhesive interactions**

(A) Coverslips were precoated with JAM-C-Fc, VCAM-1-Fc or BSA. B16 melanoma cells were perfused over these coverslips in a flow chamber using higher (1000 s⁻¹) or lower (500 s⁻¹) shear rates. After 10 min, firmly adherent cells were quantified by offline counting. The mean+/−S.E.M. (n=4-5) is shown. *, p<0.05. (B,C) In a similar fashion as under (A), B16 cells were perfused over immobilized JAM-C-Fc (B) or VCAM-1 (C) in the absence (open bars) or presence (gray bars) of smJAM-C (10 µg/mL) as indicated. Data are expressed as % of control, whereby adhesion in the absence of smJAM-C represents the 100% control. The mean+/−S.E.M. (n=3) is shown. *, p<0.05. (D) 96-well plates were precoated with JAM-C-Fc, VCAM-1-Fc or fibronectin (FN) (10 µg/mL each). B16 cells (5x10⁴/well) transfected with control siRNA or JAM-C-targeting siRNA, were allowed to adhere to these plates. The mean+/−S.E.M. (n=3) is shown. *, p<0.05. (E) CHO cells were transfected with empty vector, wildtype JAM-C (64-66 RIE) or mutant JAM-C isoforms E66A (64-66 RIA) and E66R (64-66 RIR). The adhesion of B16 cells to the different surface-adherent CHO transfectants is presented. The mean+/−S.E.M. (n=3) is shown. *, p<0.05 as compared to adhesion to vector-transfected cells.
**Fig.3: Impact of JAM-C on transendothelial migration of B16 cells**

(A, B) Murine endothelial cells (b.End.3) were grown to confluence on transwell chambers. B16 cells expressing CXCR4 (2x10^5/well) were added to the upper well and allowed to migrate towards SDF-1alpha in the lower well. (A) Prior to the transmigration experiment, B16 cells were transfected with control siRNA or siRNA targeting JAM-C. (B) Prior to the transmigration experiment, b.End.3 cells were transfected without (-) or with control siRNA or siRNA targeting JAM-C. In (A), (B) the mean+/-S.E.M. (n=3) is shown. *, p<0.05.

**Fig. 4: Inactivation of JAM-C in mice**

(A) Real time PCR analysis for JAM-C mRNA expression in lung tissues from JAM-C^+/+ (wt), JAM-C^-/- (ko), JAM-C^+/- (het) and JAM-C^lox/lox (floxed) mice (n=4 per group). (B) Conditional inactivation of JAM-C in endothelial cells through tamoxifen inducible Scl-Cre-ERT. FACS staining for JAM-C expression in primary mouse lung endothelial cells isolated from Scl-CreERT^+:JAM-C^floxed/floxed or Scl-CreERT^-:JAM-C^floxed/floxed mice that were treated with or without 4-hydroxytamoxifen (4-OHT). 4-OHT was added to the cell culture medium after the second purification step of primary endothelial cells and FACS for JAM-C expression was performed after 72 h. Representative staining of Scl-CreERT^+:JAM-C^floxed/floxed or Scl-CreERT^-:JAM-C^floxed/floxed mouse endothelial cells with isotype control (□), or JAM-C specific antibody in the absence (□) or presence (■) of 4-OHT. (C) Primary mouse lung endothelial cells isolated from Scl-CreERT^+:JAM-C^floxed/floxed mice treated without (control) or with 4-OHT to induce JAM-C deletion were grown to confluence in the upper well of transwell chambers. B16 cells expressing CXCR4 were added to the upper well and allowed to migrate towards SDF-1alpha in the lower well. The number of transmigrated cells is shown as the mean+/-S.E.M. (n=3). *, p<0.05.
**Fig. 5: JAM-C mediates melanoma lung metastasis in vivo**

(A, B) Luciferase-expressing B16 cells were injected into the tail vein of littermate JAM-C-sufficient (WT) and JAM-C-deficient mice backcrossed into the C57BL/6 background for 7 generations. (A) After 21 days, animals were sacrificed, and luciferase activity in the lungs was detected as a measure of metastasis. Data are shown as mean+/−S.E.M (n=4-5 mice per group) and are expressed as percent of control; the luciferase activity (relative light units; RLU) of WT mice represents the 100% control. *, p<0.05. (B) CT scan images of WT or JAM-C−/− mice are shown (white arrows indicate metastasis).

**Fig. 6: Endothelial-specific JAM-C deletion results in reduced melanoma lung metastasis in vivo**

(A) Representative FACS staining for CD31 expression or control antibody staining in primary mouse lung endothelial cells after the second purification step; approximately 90% of the cells were CD31-positive. Real-time PCR analysis for JAM-C mRNA expression in primary endothelial cells isolated from VEC-Cre−:JAM-Cfloxl/+; VEC-Cre−:JAM-Cfloxl/− or VEC-Cre+:JAM-Cfloxl/− mice revealed the efficient deletion of JAM-C in endothelial cells. JAM-C expression was normalized to Hprt as a housekeeping gene. (B) Luciferase-expressing B16 cells were injected into the tail veins of littermate endothelial-specific JAM-C-sufficient (VEC-Cre−:JAM-Cfloxl/+ and VEC-Cre+:JAM-Cfloxl/−) or JAM-C-deficient (VEC-Cre+:JAM-Cfloxl−) mice. After 21 days, animals were sacrificed, and lung metastasis was analyzed by measuring luciferase activity. Data are shown as mean+/−S.E.M (n=4-5 mice per group) and are expressed as percent of control; the luciferase activity (relative light units; RLU) of VEC-Cre−:JAM-Cfloxl/+ mice represents the 100% control. *, p<0.05. (C) WT mice were injected on day 0, 3, 7 and 10 with either smJAM-C or control peptide and metastasis was assessed 14 d after
injection of tumor cells. Data are shown as mean+/-S.E.M (n=7 mice per group) and are expressed as percent of control; luciferase activity (relative light units; RLU) of control-treated mice represents the 100% control. *, p<0.05.
Langer et al., Fig 1

A

benign  malignant  metastatic

B

Color intensity per area

benign  malignant  metastatic

*
Figure 3

A

Number of transmigrated B16 cells

Control-siRNA  JAM-C-siRNA

B

0  50  100  150  200  250

Control-siRNA  JAM-C-siRNA

* p < 0.05

Legend:

- Control-siRNA
- JAM-C-siRNA
A

Langer et al., Fig 4

Relative cDNA expression
JAM-C/Hprt

wt  ko  het  fl/fl

B

Cre+ JAM-C fl/fl

Cre- JAM-C fl/fl

C

Number of transmigrated B16 cells

control  4-OHT

*
Langer et al., Fig 5

A

Luciferase activity (RLU)

(% of control)

WT

JAM-C-/-

B

WT

JAM-C-/-
A

Langer et al., Fig 6

1.4
0.4
0.6
0.8
1
1.2

Relative cDNA expression
JAM-CHPRT

Cre- fl/+  Cre- fl/-  Cre+ fl/-

Control
CD31

0 200 400 600 800 1000
FSC-Height

0 200 400 600 800 1000
FSC-Height

B

Luciferase activity (RLU)
(% of control)

0 40 80 120

Cre- fl/+  Cre- fl/-  Cre+ fl/-

C

Luciferase activity (RLU)
(% of control)

0 20 40 60 80 100

control  smJAM-C
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