Targeting Olfactomedin-like 3 Inhibits Tumor Growth by Impairing Angiogenesis and Pericyte Coverage

Marijana Miljkovic-Licina, Philippe Hammel, Sarah Garrido-Urbani, Boris P.-L. Lee, Mehdi Meguenani, Chiraz Chaabane, Marie-Luce Bochaton-Piallat and Beat A. Imhof

Authors` Affiliations: Department of Pathology and Immunology, CMU, University of Geneva, Switzerland

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Corresponding Author: Beat A. Imhof, Department of Pathology and Immunology, CMU, rue Michel-Servet 1, CH-1211 Geneva 4, Switzerland. Phone: +41 22 379 57 47; Fax: +41 22 379 57 46; E-mail: beat.imhof@unige.ch.

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Abstract

Anti-angiogenic drugs have been used as anti-cancer agents to target tumor endothelial cells or pericytes. Due to limited efficacy of the current mono-therapies, there is a strong demand for dual targeting of endothelial cells and pericytes. Here, we identify Olfactomedin-like 3 (Olfml3) as a novel pro-angiogenic cue within the tumor microenvironment. Tumor-derived Olfml3 is produced by both tumor endothelial cells and accompanying pericytes and deposited in the peri-vascular compartment. Blockade of Olfml3 by anti-Olfml3 antibodies is highly effective in reducing tumor vascularization, pericyte coverage and tumor growth. In vitro, Olfml3 targeting is sufficient to inhibit endothelioma cell migration and sprouting. Olfml3 alone or through binding to BMP4 enhances the canonical SMAD1/5/8 signaling pathway required for BMP4-induced angiogenesis. Therefore, Olfml3 blockade provides a novel strategy to control tumor growth by targeting two distinct cell types within the tumor microenvironment using a single molecule.
Introduction

Tumor growth is dependent on angiogenesis, the growth of new blood vessels from the pre-existing vasculature into the tumor mass (1, 2). The tumor microenvironment contributes to this pathological vascularization process via a complex network of extracellular matrix molecules and various cell types (3). Most cancer therapies are directed against tumor cells, however a growing number of cancer therapeutics targets cells of the tumor microenvironment (4). Particularly, endothelial cells are targeted via inhibition of the vascular endothelial growth factor (VEGF) signaling pathway, but with limited long-term benefits and eventual development of resistance to the therapy (5). The resistance is due to various mechanisms, including upregulation of alternative pro-angiogenic signaling pathways, enhanced invasive/metastatic activity of tumor cells, increased recruitment of pro-angiogenic bone marrow-derived cells to tumor, and increased pericyte coverage to render tumor endothelial cells more resistant to anti-angiogenic therapy (6). Therefore, new anti-angiogenic drug targets aimed at cells of the tumor microenvironment are necessary to complement existing therapies.

A growing body of evidence suggests that bone morphogenetic proteins (BMPs) are critical growth factors in the endothelial adaptation associated with tumor progression (7). BMPs are the largest subgroup of the transforming growth factor-β super family of signaling molecules. Although initially characterized by their ability to induce ectopic bone formation (8), these proteins were recently demonstrated to be principal regulators of blood vessel formation (9). BMPs, notably BMP2 and BMP4, are pro-angiogenic factors via induction of VEGF expression (10) and recruitment of endothelial progenitor cells (11). The pro-angiogenic effects of BMP4 are mediated via activation of VEGF/VEGFR2 and angiopoietin/Tie2 signaling (12). However,
endothelial migration and sprouting can also be regulated through canonical BMP signaling independently of VEGF (13, 14). In addition to autocrine effects on tumor cells, a paracrine effect of BMP4 on the vascular network has also been reported. Notably, melanoma cell-derived BMP4 induces endothelial cell migration and tube formation (15). During progression of hepatocellular carcinoma, hypoxia-induced BMP4 mediates endothelial cell activation (16). Therefore, the BMP4-induced angiogenic response involves several mechanisms.

The *Olfactomedin-like 3 (Olfml3)* gene encodes a secreted extracellular protein, also known as ONT1 in *Xenopus* and chicken, mONT3 in mice, and HNOEL-iso or hOLF44 in humans (17-20). Olfml3 belongs to a family of olfactomedin domain-containing proteins with distinct roles in embryonic patterning, cell cycle regulation and tumorigenesis, and ability to modulate critical signaling circuits like Notch or Wnt pathways (21, 22). *Xenopus* ONT1 serves as a scaffold protein that recruits the BMP1/Tolloid proteases to their substrate chordin, a BMP antagonist (19). This function of Olfml3 may prove relevant to angiogenesis. However, an interaction of Olfml3 with other BMPs, particularly those with prominent pro-angiogenic activity in tumors like BMP4, has not been demonstrated.

Here, we provide the first report on Olfml3 protein being a pro-angiogenic factor that interacts with BMP4 and promotes angiogenesis. We also demonstrate that inhibition of Olfml3 in tumor endothelial cells and pericytes effectively reduces tumor angiogenesis and growth *in vivo*. 
Materials and Methods

Cell lines and culture

t.End.1V\textsuperscript{high} cells were isolated in-house and maintained as described previously (23). Authenticated Lewis lung carcinoma cells (LLC1; European Collection of Cell Cultures) were cultured in DMEM (Life Technologies), supplemented with 10% FBS. Smooth muscle cells (SMCs) were isolated in-house from porcine carotid artery using enzymatic digestion (S-SMCs) or tissue explantation (R-SMCs) as described previously (24). HUVECs were isolated in-house and cultured in EGM-2 Bulletkit (Lonza).

Tumor model

All studies were conducted in accordance with the ethical approval and recommendations of the Veterinary Office of Geneva state, according to the Swiss federal law. To generate tumor growth inhibition model, a suspension of $0.5\times10^6$ LLC1 tumor cells in 200 µL PBS was implanted subcutaneously into the flank of female C56BL/6J mice (8-10 weeks old). Mice were then treated with 25-50 µg of control, total rabbit IgG; 50 µg of anti-Olfml3\textsuperscript{A+B} affinity-purified against both Olfml3 peptides, and 25 µg of anti-Olfml3\textsuperscript{A} or anti-Olfml3\textsuperscript{B} affinity-purified against each peptide i.p. every third day starting from day 1. When tumors reached an average size of 1 cm\textsuperscript{3} (day 8-9), mice were sacrificed and tumors were harvested for evaluation of tumor growth.

\textit{In situ} mRNA hybridization

The digoxigenin- and fluorescein-labeled (Roche) RNA probes were prepared after PCR amplification of mouse \textit{PECAM-1} and \textit{Olfml3} genes as described in Supplementary Methods. \textit{In situ} mRNA hybridization was performed on frozen sections of LLC1 tumors as previously described (25).
Immunohistochemistry and TUNEL assay

HUVECs were grown on glass slides and immunohistochemistry was performed as detailed in Supplementary Methods. LLC1 tumors were processed for and stained by immunohistochemistry as previously described (25). Samples were incubated with: rabbit anti-Olfml3A+B serum, rat monoclonal anti-PECAM-1 (26), mouse monoclonal anti-α-SMA (27), mouse monoclonal anti-NG2 (clone 132.38; Millipore), mouse anti-Ki-67 (BD Pharmingen) and rabbit anti-HIF-1α (Santa Kruz). For apoptotic cell labeling, the TUNEL assay (Roche) was performed according to the manufacturer’s instruction. ImageJ was used for quantification of vessel diameter and colocalization analysis as described in Supplementary Methods. Quantification of area density and pericyte coverage was performed using Metamorph6.0 (Molecular Devices) as described in Supplementary Methods.

In vitro wound-healing assay

Transient transfection of t.End.1V high cells was performed using Amaxa™-Nucleofector (Lonza) with Stealth™-Select siRNAs (Life Technologies) as described in Supplementary Methods. The efficiency of Olfml3 silencing was evidenced by RT-qPCR as described in Supplementary Methods. t.End.1V high cells (1.5×10^4) and R-SMCs (8×10^4) were seeded onto rOlfml3-FLAG-coated (BD Biosciences) plates and wound-healing assays were performed as described previously (25).

In vitro sprouting assay

t.End.1V high cells (1.2×10^4 cells/gel) were seeded in suspension into fibrin gels (28) and sprouting assays were performed as described previously (25).

Enzyme-linked immunosorbent assay (ELISA)

Maxisorb immunoplates (Nunc) were coated with rBMP4 (2 μg/mL). Wells were blocked with 1% BSA and incubated with rOlfml3-FLAG at 0.5 μg/mL in PBS
containing 0.05% Tween 20 and 0.5% BSA. Biotinylated-M2 antibody (2 µg/mL) was added. Bound M2 was detected using streptavidin-HRP (Jackson Immunoresearch Laboratories) and substrate Reagent Pack (R&D Systems). Optical densities at 450 nm were read using a kinetic microplate reader and SoftMAXPro (Molecular Devices).

**Pull-down assay of rBMP4 by rOlfml3-FLAG**

rBMP4 (R&D Systems) was incubated with anti-FLAG M2-Agarose beads (Sigma-Aldrich) loaded with or without rOlfml3-FLAG (1 µg) in TBS, 0.1% NP-40, 0.05% BSA. Beads were eluted with non-reducing SDS sample buffer. Samples were subjected to SDS-PAGE and silver staining was performed using SilverQuest (Invitrogen).

**Western blotting**

HUVECs were serum-starved in OptiMEM (Invitrogen) and 50 ng/mL rBMP4 (R&D Systems) and/or 50 ng/mL of rOlfml3-FLAG were added. Cells were lysed with lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl2 and 0.5% Triton X-100] containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich). Blots were incubated with anti-phosphoSMAD1/5/8 or anti-SMAD1 (Cell Signaling) and revealed using the HRP-labeled anti-rabbit antibodies (Jackson Immunoresearch Laboratories), visualized using an enhanced chemiluminescence system and a quantitative imaging system LAS4000Mini (Fujifilm).

**Statistical analysis**

All data are presented as means ± standard deviation (SD) unless indicated otherwise. For comparisons of two means, Student's t-test (2-sided, paired) was used. For multiple mean comparisons, one-way or two-way ANOVA followed by the
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Bonferroni’s test was used. All computations were done using GraphPadPrism. Results were considered statistically significant at P<0.05.
Results

Dual Expression of Olfm3 in Tumor Endothelium and Accompanying Pericytes

To mimic molecular and functional properties of endothelial cells during tumor angiogenesis, we previously isolated two subpopulations of an endothelioma cell line with molecular characteristics of angiogenic (t.End.1Vhigh) and resting (t.End.1Vlow) cells (23). The t.End.1Vhigh cells expressed high levels of the integrin αVβ3 and did not endocytose acetylated low-density lipoprotein (Ac-LDL), while t.End.1Vlow cells expressed low levels of αVβ3 integrin and efficiently endocytosed Ac-LDL. Additionally, t.End.1Vhigh cells showed increased migration and sprouting in three dimensional (3D)-fibrin gels (23). These cells were exploited as a starting point for the transcriptomic profiling that yielded several new candidate genes without previously known pro-angiogenic activity (25). One of the most promising candidate genes in this category was Olfm3, since its expression was found to be restricted to t.End.1Vhigh cells (Supplementary Fig. S1A). Accordingly, in vivo Olfm3 expression was detected on vessels undergoing angiogenesis in matrigel plugs (Supplementary Fig. S1B). To evaluate Olfm3 expression in tumor vessels, Lewis Lung Carcinoma (LLC1) cells were s.c. implanted in wild-type mice (Fig. 1). While PECAM-1 transcripts were abundantly expressed along the tumor endothelium, Olfm3 transcripts were expressed by a subset of endothelial cells (PECAM-1+) and vessel-associated pericytes (PECAM-1-) (Fig. 1A). Tumor cells themselves did not express Olfm3 mRNA (Supplementary Fig. S1C). Double staining of tumors for Olfm3 and PECAM-1 revealed that Olfm3 protein is enriched in the extracellular space of endothelial cells and pericytes of a subset of tumor vessels (Fig. 1B). To validate vascular-specific Olfm3 expression, tumors were triple-stained for Olfm3, PECAM-1,
and the pericyte markers α-smooth muscle actin (α-SMA) or nerve/glial antigen-2 (NG2), respectively (Fig. 1C, D). Olfml3 expression was detected in both α-SMA+ and NG-2+ pericytes of PECAM-1+ vessels, while it was absent from PECAM-1+ vessels not covered by α-SMA+ cells (Fig. 1C). Two-color images of LLC1 tumor sections were used to quantify colocalization of Olfml3 and PECAM-1, α-SMA or NG2 staining respectively (Fig. 1E). We observed that ∼25% of Olfml3 colocalized with either PECAM-1 or α-SMA, while ∼55% colocalized with NG2 (Fig. 1E). These data illustrated that endogenous Olfml3 is expressed by both tumor endothelial cells and pericytes. In order to determine whether Olfml3 is produced by pericytes embracing established tumor vessels or de novo-forming vessels, we isolated two distinct smooth muscle cell (SMC) populations having pericyte-like characteristics: resting, spindle shaped (S-SMC) and activated, rhomboid (R-SMC) cells (24). R-SMCs displayed enhanced proliferative, migratory and proteolytic activities and were less differentiated compared with S-SMCs (24, 29). Activated R-SMCs expressed significantly higher levels of Olfml3 compared with S-SMCs (Fig. 1F). Therefore, Olfml3 expression levels correlate with the activation state of both endothelial cells and pericytes, implying a potential functional importance of Olfml3 during activation and maturation phases of angiogenesis.

**Autocrine Effects of Olfml3 on Endothelial cells and R-SMCs**

To define the Olfml3-dependent vascular functions, we first tested whether Olfml3 mediates endothelial and pericyte cell migration. As t.End.1Vhigh cells migrate efficiently in wound-healing assays (23, 25), we investigated the consequences of Olfml3 gene silencing (Supplementary Fig. S2A) on the migration of t.End.1Vhigh cells in this assay. The Olfml3-silenced t.End.1Vhigh cells displayed a significantly
decreased migration rate into the denuded area (Fig. 2A). Olfml3 silencing did not affect endothelial cell proliferation (not shown). This reduced migratory ability of Olfml3-silenced cells was partly compensated when recombinant Olfml3-FLAG-tagged protein (rOlfml3-FLAG) (Supplementary Fig. S2B) was coated on plates (Fig. 2B). Conversely, t.End1V\textsuperscript{high} cells treated with anti-Olfml3\textsuperscript{A+B} antibody migrated less efficient than cells treated with control rabbit immunoglobulin G (IgG) (Supplementary Fig. S2C). Correspondingly, rOlfml3-FLAG promoted t.End.1V\textsuperscript{high} cell migration compared with control FLAG peptide or ΔJAM-C-FLAG (Fig. 2C). The rOlfml3-FLAG also promoted migration of R-SMCs (Fig. 2D) and had no effect on their proliferation (not shown). These data identified Olfml3 as a novel autocrine regulator of endothelial and pericyte-like cell migration.

The pro-migratory action of Olfml3 on t.End.1V\textsuperscript{high} cells suggested that Olfml3 might also exert an effect on endothelial cell sprouting. As t.End.1V\textsuperscript{high} cells form a capillary-like network of ramified cords in 3D-fibrin gels (23), we used this assay to study the effect of Olfml3 depletion on t.End.1V\textsuperscript{high} cell sprouting (Fig. 2E-G). Compared with mock- or control siRNA-treated t.End.1V\textsuperscript{high} cells (Fig. 2E), the number of Olfml3-silenced cells that initialized sprout protrusions at early time points (24-32h) was significantly decreased (Fig. 2E, F). In addition, total length of the vascular network in Olfml3-silenced cells was reduced at later time points (72h) (Fig. 2G and Supplementary Fig. S3A). Accordingly, the number of sprouting cells (24h) and total length of the vascular network (48h) of anti-Olfml3\textsuperscript{A+B}-treated cells were reduced compared with control IgG-treated cells (Supplementary Fig. S3B, C). Anti-Olfml3 antibodies had no effect on t.End1V\textsuperscript{high} cells that have already sprouted (72h) (Supplementary Fig. S3C). These findings suggest that abrogation of Olfml3 was
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sufficient to attenuate endothelial cell sprouting, further supporting its potential role in angiogenesis.

Anti-Olfml3 Antibodies Reduce LLC1 Tumor Growth and Angiogenesis

In order to test whether Olfml3 promotes tumor angiogenesis in vivo, we generated rabbit anti-Olfml3 antibodies by injecting simultaneously two 13-aa long peptides comprising epitopes in the coiled-coil (peptide A) and the olfactomedin-like domains (peptide B) (Supplementary Fig. S4A). Both peptides are identical in the mouse and human Olfml3 protein sequences (Supplementary Fig. S4B). The anti-Olfml3 antibodies recognized the peptides A and B, respectively (Supplementary Fig. S4C) and rOlfml3-FLAG (Supplementary Fig. S2B).

The Olfml3 antibodies were affinity-purified against both Olfml3 peptides (anti-Olfml3A+B) and evaluated for the ability to block tumor growth and angiogenesis in the LLC1 mouse model. Treatment with anti-Olfml3 A+B antibodies significantly decreased the tumor weight compared with control rabbit IgG treatment (Fig. 3A). To determine which Olfml3 structural domain might be necessary for this effect, we affinity-purified the Olfml3 antibodies against either the Olfml3 peptide A (anti-Olfml3A) or peptide B (anti-Olfml3B) and used them for tumor treatment. Both antibodies significantly reduced tumor growth by 38% and 52% respectively with no significant difference observed between either treatment (Fig. 3B). The rate of tumor vascularization measured by PECAM-1+ area density was decreased by treatment with either anti-Olfml3A or anti-Olfml3B (Fig. 3C, D). The antibodies showed different efficacy of reducing tumor vascularization. Anti-Olfml3B reduced tumor vascularization by 55%, whereas anti-Olfml3A had smaller but still significant effect (25%) (Fig. 3D), suggesting that both structural domains of the protein are necessary for its pro-
angiogenic activity. However, when the two Olfml3 antibodies were co-injected, no synergistic inhibition of tumor vascularization was observed (not shown). To further analyze the vascular effects of Olfml3 targeting, we determined average vessel diameter in tumors of control- and anti-Olfml3-treated mice. This quantitative assessment revealed that anti-Olfml3-mediated treatment led to smaller, collapsed vessels (Fig. 3E) and thus, correlated to the decrease of vessel density (Fig. 3D). These findings confirmed our hypothesis that Olfml3 promotes tumor angiogenesis, whereas blocking its function leads to reduced angiogenesis and tumor growth.

As tumor growth is the net result of tumor cell proliferation and apoptosis, we next determined the proliferative and apoptotic index of tumors using Ki-67 immunostaining and the TUNEL assay respectively (Fig. 3F, G). Neither anti-Olfml3A nor anti-Olfml3B treatment significantly affected the number of proliferating Ki-67+ cells (Fig. 3F). In contrast, anti-Olfml3B-mediated treatment significantly increased the number of TUNEL+-apoptotic cells compared with control or anti-Olfml3A-treated tumors (Fig 3G). This suggests that anti-Olfml3B treatment leads to increased cell death, thus explaining decreased tumor growth. In order to determine the level of tumor hypoxia, we next assessed activity of hypoxia inducible factor-1α (HIF-1α) in control- and anti-Olfml3-treated tumors (Fig. 3H). Tumors of control-treated mice showed the translocation of HIF-1α into the nucleus and to perinuclear areas (Fig. 3H). Both anti-Olfml3A- and anti-Olfml3B-treated tumors did not have HIF-1α-immunoreactive cells (Fig. 3H). This suggests that anti-Olfml3 treatment does not induce massive hypoxia considered as detrimental effect selecting for tumor cell invasion and metastasis (3).
Endothelial cell survival correlates with the extent of pericyte coverage in tumor vessels (30). As Olfml3 was co-expressed in tumor endothelial cells and pericytes (Fig. 1), we investigated whether anti-Olfml3-mediated treatment affects pericyte density and coverage using PECAM-1, α-SMA and NG2 as the readout. Numerous α-SMA⁺ and NG2⁺ pericytes were observed under control conditions (Fig. 4A, C). Following anti-Olfml3-mediated treatment, α-SMA⁺- and NG2⁺-pericyte density decreased by ∼40% and ∼25% respectively (Fig. 4B, D). Correspondingly, anti-Olfml3A- or anti-Olfml3 B-mediated treatment reduced NG2⁺-pericyte coverage by ∼40% (Fig. 4E). These observations indicate that targeting Olfml3 decreases the pericyte coverage in tumor vessels, implying Olfml3 involvement in the maturation of de novo-forming vasculature.

**Olfml3 is a BMP4-binding Protein**

Previous studies have shown that *Xenopus* Olfml3 interacts with BMP1 and chordin through the coiled-coil and olfactomedin-like domains, respectively (19). We therefore investigated a possible interaction of Olfml3 with BMPs known as either pro- or anti-angiogenic cues within the tumor microenvironment (31). We used rOlfml3-FLAG for interaction studies with three different BMPs in enzyme-linked immunosorbent assays (ELISA). rOlfml3-FLAG specifically bound recombinant BMP4 (rBMP4) but not rBMP1 or rBMP9 (Fig. 5A), and rOlfml3-FLAG co-immunoprecipitated with rBMP4 (Fig. 5B). To map the BMP4-binding regions on the Olfml3 protein, anti-Olfml3A, anti-Olfml3 B and a commercial antibody raised against a distinct Olfml3 peptide (Olfml3 peptide C) were used for binding studies (Fig. 5C). Both anti-Olfml3A and anti-Olfml3 B antibodies partially blocked the interaction of rOlfml3-FLAG with rBMP4 (Fig. 5D). The third high-affinity antibody, targeting a non-
overlapping epitope in the coiled-coiled domain, did not block Olfml3-BMP4 interaction (Fig. 5E). These results suggest that the coiled-coil (peptide A) and the olfactomedin-like domain (peptide B) are equally required for the interaction with BMP4, confirming our previous hypothesis of a single ligand for the two Olfml3 domains. Our results define a novel interaction between mouse Olfml3 and BMP4, a potent pro-angiogenic growth factor.

**Olfml3 Activates Canonical SMAD1/5/8 Signaling Pathway in HUVECs**

As BMP4 directly binds to Olfml3 (Fig. 5), we sought to investigate the possible effect of this interaction in BMP4 downstream signaling. HUVECs were treated with rOlfml3-FLAG and/or BMP4 and subsequently both nuclear translocation of SMAD1 and phosphorylation of SMAD1/5/8 as readouts of the BMP4 pathway activity were analyzed (Fig. 6). rOlfml3-FLAG alone induced nuclear translocation of SMAD1 after 15 minutes (Fig. 6A). Likewise, nuclear translocation of SMAD1 was observed in BMP4-treated HUVECs (Fig. 6A). Upon challenge of HUVECs with rOlfml3-FLAG or BMP4, Smad1/5/8 proteins were phosphorylated rapidly (Fig. 6B-D), whereas SMAD1/5/8 phosphorylation was not observed in untreated control cells (data not shown) or cells treated with the FLAG peptide (Fig. 6B, C). In the presence of anti-Olfml3A+B antibodies, the ability of Olfml3 to induce SMAD1/5/8 phosphorylation is lost (Fig. 6B, C). Of interest, Olfml3 and BMP4 showed additive effects on pSMAD1/5/8 phosphorylation when combined (Fig. 6B-D). While SMAD1/5/8 phosphorylation reached a maximum after 15 minutes of rOlfml3-FLAG exposure in HUVECs (Fig. 6D), rOlfml3-FLAG and BMP4 exposure gave rise to an increased and prolonged effect on SMAD1/5/8 phosphorylation in time course experiments (Fig. 6C, D). These findings demonstrate that Olfml3 alone or in a complex with BMP4 acts as an enhancer of the SMAD1/5/8 signaling pathway in HUVECs.
Discussion

Olfml3 has been recently described as an extracellular modulator of BMP signaling during embryogenesis (19). BMP4 signaling is shown to be critically involved in the development of blood vessels during embryogenesis and adult life (7, 9). To date, there have been no studies demonstrating Olfml3 ability to regulate adult vascular remodeling under normal or pathological conditions. Herein, we show that Olfml3 expression is restricted to vessels undergoing angiogenesis as shown in matrigel plugs and tumors. Olfml3 is expressed by angiogenic endothelial cells and pericytes and deposited in the perivascular compartment. Our findings provide a paradigm for the contribution of a novel vascular cue within the tumor microenvironment that reinforces tumor growth.

Intense α-SMA and NG2 staining in pericytes characterize the phenotype of mature blood vessels (32). Here we show that Olfml3 expression is concomitant with α-SMA and NG2 staining in tumor vessel-associated pericytes. In addition, we found that both α-SMA and NG2-expressing pericytes were depleted in tumor-bearing mice that are treated with anti-Olfml3 antibodies. Part of this depletion may reflect reduced pericyte proliferation, but it is also possible that pericyte migration along the new tumor vessels is impaired after anti-Olfml3 treatments. In support of this, we observed that Olfml3 promotes migration of activated pericyte-like cells (24). Therefore, Olfml3 may contribute to a pro-angiogenic microenvironment that supports remodeling and maturation of tumor vessels. Accordingly, anti-Olfml3 tumor treatments substantially affect both angiogenic endothelium and accompanying pericytes. In comparison, anti-VEGF treatments generally eliminate tumor vessels without removing pericytes (33). Upon cessation of anti-VEGF therapy, empty sleeves of basement membrane and accompanying pericytes provide a scaffold for
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rapid revascularization of tumors (34). Of interest, increased and tight pericyte coverage of newly formed vessels contributes to the resistance of tumors to anti-VEGF therapy (6). Thus, targeting Olfml3 has a potential advantage over anti-VEGF therapy by reducing tumor growth while simultaneously affecting tumor endothelium and pericytes. In addition, targeting perivascular Olfml3 may affect an intricate crosstalk between endothelial cells and pericytes. It is now widely accepted that pericytes limit the effectiveness of anti-angiogenic therapy by providing survival signals for endothelial cells (35). However, inhibition of PDGFRβ signaling, which is critical for pericyte recruitment, reduces pericyte coverage but has limited efficacy on endothelial cell regression (36, 37). Inhibition of PDGFβ signaling can indirectly reduce tumor vascularization, but does not necessarily retard tumor growth (38).

Several studies have shown that dual targeting of endothelial cells and pericytes is more efficient than targeting either cell type alone, even in established or drug-resistant tumors (39-41). Therefore, blocking Olfml3 holds promise for more effective strategies to control tumor growth by targeting a single molecule that affects two distinct cell types within the tumor microenvironment.

Two different Olfml3 antibodies, recognizing either an epitope in the coiled-coil or in the olfactomedin-like domain, substantially inhibited tumor growth and angiogenesis. We did not observe a synergistic effect of the two antibodies, implying that both Olfml3 epitopes are functional and equally necessary for angiogenic outcome. This is possible only if the two Olfml3 domains interact simultaneously with a putative ligand. Accordingly, our binding studies indicated that Olfml3 interacts with BMP4 and both Olfml3 antibodies were able to specifically interfere with the Olfml3-BMP4 complex. Previous studies by others have shown that Xenopus ON1 binds BMP1 and chordin through the coiled-coil and olfactomedin-like domain, respectively, whereas it does
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not interact with BMP4 (19). We show here that Olfml3 does not bind BMP1 or BMP9, both known for their anti-angiogenic activities (42, 43). Together, these findings suggest that Olfml3 creates specificity for BMP4 by two binding sites interacting simultaneously. Inhibition of Olfml3-BMP4 interaction might be a major mechanism by which anti-Olfml3 antibodies exert their anti-tumor and anti-angiogenic effects. Nonetheless, we cannot dismiss the possibility that the reduction in tumor growth by anti-Olfml3B antibody was in part a consequence of affecting tumor cell function indirectly.

BMP4 itself signals to endothelial cells in an autocrine manner via the canonical SMAD1/5/8 pathway (44, 45). Our data demonstrate that Olfml3 itself promotes endothelial cell migration and sprouting and activates SMAD1/5/8 signaling, implying a role for Olfml3 in the activation of endothelial cells. In addition, Olfml3 binds BMP4 and this potentiates SMAD1/5/8 signaling. Therefore, there are at least two possible scenarios that can be proposed for Olfml3-dependent activation of endothelial cells through stimulation of the SMAD1/5/8 pathway. First, Olfml3 could facilitate cell surface retention of BMP4 to promote its interaction with receptors, thereby enhancing BMP4-induced recruitment and activation of Smad1/5/8 at the plasma membrane. Second, Olfml3 may promote BMP4 activity by dislodging BMP4 from a putative antagonist in the extracellular space (46), as shown for pro-BMP activity of Xenopus ONT1 or Twisted gastrulation (19, 47). Additional studies are needed to elucidate the definitive mode of action by which Olfml3 stabilizes BMP4 and potentiates endothelial cell signaling.

In conclusion, we provide the first evidence for Olfml3 being a novel pro-angiogenic factor, which joins an intriguing group of BMP modulators and affects angiogenesis during normal and pathological conditions. In particular, our study demonstrates the
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functional importance of Olfm13 during tumor angiogenesis, and it establishes the potential value of this extracellular molecule as a target for anti-angiogenic therapy specific for both endothelial cells and pericytes. Given the potential side effects and resistance linked to current anti-angiogenic therapies, a better understanding of novel angiogenic signaling circuits can accelerate the development of alternative and more selective strategies, which in turn may be used in combination with VEGF inhibitors to increase the efficacy of anti-angiogenic tumor treatments.
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References

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Figure Legends

Figure 1. Increased Olfml3 expression in tumor endothelial cells and pericytes. A, in situ mRNA hybridization of LLC1 tumors with Olfml3 (green) and PECAM-1 (red) RNA probes shows Olfml3 expression on tumor vessels (arrows; inset, arrowheads) and vessel-associated pericytes (insets, stars). No staining with sense Olfml3 RNA probe (sense). Olfml3-expressing endothelial cells (anti-sense) are PECAM-1⁺ (overlay). Pericytes express Olfml3 but not PECAM-1 (insets, stars). B, Olfml3 (red) and PECAM-1 (green) immunostaining of tumors shows Olfml3 expression on tumor vessels (arrows) and pericytes (overlay/inset, stars). Pericytes express Olfml3 but not PECAM-1 (insets, stars). C, Olfml3 (light blue), PECAM-1 (green) and α-SMA (red) immunostaining shows Olfml3 expression on tumor vessels and accompanying pericytes (arrows). No Olfml3 staining on PECAM-1⁺ vessels not covered by α-SMA⁺ cells (stars). D, Olfml3 (light blue), PECAM-1 (green) and NG2 (red) immunostaining shows Olfml3 expression on tumor vessels and accompanying pericytes (arrows). DAPI- nuclear counterstain (blue) (A–D, overlays). E, left: merged two-color images of LLC1 tumors show the amount of colocalization of Olfml3 and PECAM-1, α-SMA or NG2 staining (white). Right: quantification of colocalization of Olfml3 and PECAM-1, α-SMA or NG2 staining. Error bars represent ± SD. F, relative Olfml3 mRNA levels in activated R-SMCs versus resting S-SMCs. Error bars represent ± SD (2 experiments, each group in triplicates); ***P<0.001. Bars correspond to 30 µm (A, B), 20 µm (C, D) 10 µm (B, inset; E) and 5 µm (A, insets).

Figure 2. Effects of Olfml3 targeting and rOlfml3-FLAG on t.End.1Vhigh cell migration and sprouting. A, left: in vitro migration assays using mock, control siRNA (ctrl
siRNA, 0.5 µM) or Olfml3 siRNA-treated (Olfml3 siRNA, 0.5 µM) t.End.1V<sup>high</sup> cells. Confluent cell monolayers were wounded (yellow). Cells migrated into the wounded area after 16 hours (violet). Right: quantification of migration distance (µm) of mock, control- or Olfml3 siRNA-treated t.End.1V<sup>high</sup> cells. B, rescued migratory ability of Olfml3-silenced t.End.1V<sup>high</sup> cells on rOlfml3-FLAG-coated plates (1 µg/mL) when compared with non-coated control (0 µg/mL). C, coated rOlfml3-FLAG promotes t.End.1V<sup>high</sup> cell migration compared with control FLAG peptide (1 µg/mL) or ΔJAMC-FLAG (1 µg/mL). D, coated rOlfml3-FLAG promotes migration of R-SMCs compared with FLAG peptide (1 µg/mL). E, in vitro t.End.1V<sup>high</sup> sprouting assays in 3D-fibrin gels. Control siRNA-treated cells start sprouting after 24h (arrows) to form a vascular-like network (32-72h). Targeting Olfml3 delays sprouting (arrowheads) by 32h (arrows). Bars correspond to 10 µm. F, quantification of sprout-forming t.End.1V<sup>high</sup> cells at early-time points (24, 32h). Olfml3 targeting (Olfml3 siRNA) reduces the total number of sprouting cells compared with mock or control siRNA-treated cells. G, quantification of total length of vascular-like network of t.End.1V<sup>high</sup> cells treated with mock, control or Olfml3 siRNAs, normalized to total number of cells/condition. At later time points (48, 72h), targeting Olfml3 reduces the length of the vascular-like network compared with controls. Error bars represent ± SD (5 experiments; each group in triplicates; A-D, F, G). *P<0.05; **P<0.01; ***P<0.001; ns-non significant (A-D, F, G).

**Figure 3.** Inhibitory effects of anti-Olfml3 antibodies on tumor growth and vascularization. A, left: 9-day-old LLC1 tumors in mice treated with rabbit IgG (control) or anti-Olfml3<sup>A+B</sup>. Right, reduced tumor weight in mice treated with anti-Olfml3<sup>A+B</sup> compared with control. Error bars represent ± SEM (3 experiments; 4-5 mice/group; 2 tumors/mouse). *P<0.05. B, left: 9-day-old tumors in mice treated with...
control, anti-Olfml3A or anti-Olfml3B. Right, reduced tumor weight in mice treated with either anti-Olfml3A or anti-Olfml3B compared with control. Error bars represent ± SEM (2 experiments; 4-5 mice/group; 2 tumors/mouse). *P<0.05 **P<0.01, ns-non significant. C, representative images compare the dense vasculature (PECAM-1, green) of control tumors and pruned vasculature after treatments with anti-Olfml3A or anti-Olfml3B. DAPI- nuclear counterstain (blue). D, quantification of vessel density in control, anti-Olfml3A- or anti-Olfml3B-treated tumors measured as a ratio of the total pixel count of PECAM-1 to DAPI and normalized to control. E, quantification of average vessel diameter in control, anti-Olfml3A- or anti-Olfml3B-treated tumors. F, left: tumor cell proliferation (Ki67, green) in control-, anti-Olfml3A- or anti-Olfml3B-treated tumors. Right: quantification of Ki67+ cells normalized to DAPI. G, left: level of apoptosis (TUNEL, green) in control-, anti-Olfml3A or anti-Olfml3B-treated tumors. Right: quantification of TUNEL+ cells normalized to DAPI. H, level of hypoxia (HIF-1α, red) in control-, anti-Olfml3A- or anti-Olfml3B-treated tumors. Five images at three planes analyzed in 8-10 tumors/group (D, E; right: F, G). Error bars represent ± SEM (2 experiments; 4-5 mice/group; 2 tumors/mouse) (D, E; right: F, G). *P<0.05; **P<0.01; ***P<0.001; ns-non significant (D, E; right: F, G). Bars correspond to 1 cm (A, B) and 20 μm (C, F-H).

Figure 4. Anti-Olfml3 antibody tumor treatment inhibits pericyte association with vessels. A, top: the abundance of pericytes (α-SMA, red) in control-, anti-Olfml3A- or anti-Olfml3B-treated tumors. Bottom: insets of top panels at higher magnification. B, quantification of α-SMA+ area density in control-, anti-Olfml3A- or anti-Olfml3B-treated tumors measured as a ratio of the total pixel count of α-SMA to PECAM-1 and normalized to control. C, top: the abundance of pericytes (NG2, red) in control-, anti-Olfml3A- or anti-Olfml3B-treated tumors. Bottom: insets of top panels at higher magnification.
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magnification. DAPI (blue)- nuclear counterstain (A, C). D, NG2\(^+\) area density in control-, anti-Olfml3\(^A\)- or anti-Olfml3\(^B\)-treated tumors measured as a ratio of the total pixel count of NG2 (red) to PECAM-1 (green) and normalized to control. E, quantification of pericyte coverage in control-, anti-Olfml3\(^A\)- or anti-Olfml3\(^B\)-treated tumors measured as a ratio of the total pixel count of overlapping NG2/PECAM-1 to PECAM-1. Ten individual images at three planes analyzed in 8-10 tumors/group (B, D, E). Error bars represent ± SEM (2 experiments; 4-5 mice/group; 2 tumors/mouse; B, D, E). *P<0.05; **P<0.01; ns-non significant (B, D, E). Bars correspond to 20 μm (top; A, C) and 10 μm (bottom; A, C).

**Figure 5.** Recombinant Olfml3 binds rBMP4. A, binding of rOlfml3-FLAG to rBMP4 detected by ELISA using FLAG (M2) antibody. The rOlfml3-FLAG specifically recognizes BMP4 but not BMP1 or BMP9 in a dose-dependent manner (0.1–1 μg/mL). Human JAM-C-FLAG- negative control (0.1 μg/mL). B, immobilized rOlfml3-FLAG on M2-beads binds rBMP4. Silver-stained SDS gel: left, input of rBMP4 loaded for comparison (rBMP4; 21 kDa); middle, pull-down of rBMP4 by M2-beads; right, pull-down of rOlfml3-FLAG and rBMP4 by M2-beads (arrow). C, Olfml3 domains relative to anti-Olfml3\(^A\), anti-Olfml3\(^B\) and commercial anti-Olfml3\(^C\) epitope regions. D, blocking of rOlfml3-FLAG binding to rBMP4 by anti-Olfml3\(^{A+B}\) (A+B), anti-Olfml3\(^A\) (A) or anti-Olfml3\(^B\) (B), but not by rabbit IgG (control). E, blocking of rOlfml3-FLAG binding to rBMP4 by anti-Olfml3\(^{A+B}\), but not by rabbit IgG (control) or anti-Olfml3\(^C\). Error bars represent ± SD (5 experiments; each group in triplicates; D, E). *P<0.05; ***P<0.001; ns-non significant (D, E).

**Figure 6.** Olfml3 activates the canonical SMAD1/5/8 pathway. A, Olfml3 induces nuclear translocation of SMAD1/5/8. SMAD1 (red) immunostaining compares SMAD1 cytoplasmic localization in control HUVECs with SMAD1 nuclear
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translocation in HUVECs treated with rOlfml3-FLAG (Olfml3; 100 ng/mL); rBMP4 (BMP4; 50 ng/mL), or combination (Olfml3+BMP4) for 15 minutes. B, Olfml3 induces SMAD1/5/8 phosphorylation in HUVECs. PhosphoSMAD1/5/8 immunostaining (red) compares levels of phoshosMAD1/5/8 in control HUVECs (control: FLAG peptide) and levels of phosphoSMAD1/5/8 in HUVECs treated with rOlfml3-FLAG (Olfml3; 100 ng/mL), rBMP4 (BMP4; 50 ng/mL) or combination (Olfml3+BMP4) for 15 min. Olfml3 does not induce phosphoSMAD1/5/8 in the presence of anti-Olfml3A+B (Olfml3+anti-Olfml3) compared with control (Olfm3+IgG). FITC-phalloidin (green) allows visualization of the cell scaffolds (A, B). DAPI (blue)- nuclear counterstain (A, B). Scale bars represent 10 μm (A, B). C, quantification of the intensity of nuclear phosphoSMAD1/5/8 signals. rOlfml3-FLAG and rBMP4 combination (Olfml3+BMP4) shows an additive effect on SMAD1/5/8 phosphorylation. Mean nuclear intensity was measured from 5-10 fields/group in 2 experiments. ***P<0.001; ns-non significant. D, prolonged effect on SMAD1/5/8 phosphorylation using both recombinant proteins (Olfml3+BMP4), compared with the effect of rOlfml3-FLAG alone. HUVECs were treated with control (0 min); rOlfml3-FLAG (100 ng/mL) or rOlfml3-FLAG and rBMP4 (Olfml3+BMP4; 100 and 50 ng/mL, respectively) for 15, 30 and 45 minutes.
Figure 1.
Figure 2.

A

mock
ctrl siRNA
Olfml3 siRNA

B

Distance moved (µm)

mock ctrl siRNA Olfml3 siRNA

C

Distance moved (µm)

FLAG ΔJAM-C Olfml3

D

Distance moved (µm)

E

Sprouting assay

ctrl siRNA

Olfml3 siRNA

F

Number of sprout forming cells (%)

24 h 32 h

G

Total length of vascular cords /no. of cells (relative units)

48 h 72 h

mock ctrl siRNA Olfml3 siRNA

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Figure 4.

A

Control | Anti-Olfm3A | Anti-Olfm3B
LLC1: PECAM-1/α-SMA/DAPI

B

α-SMA area density (% of control)

Control | Anti-Olfm3A | Anti-Olfm3B

C

Control | Anti-Olfm3A | Anti-Olfm3B
LLC1: PECAM-1/NG2/DAPI

D

NG2 area density (% of control)

Control | Anti-Olfm3A | Anti-Olfm3B

E

Pericyte coverage (Overlapping NG2/PECAM-1) (%)

Control | Anti-Olfm3A | Anti-Olfm3B

ns

ns
Figure 5.

A

B

C

D

E
Figure 6.

A

HUVEC: 15 min

Control | Olfm3 | BMP4 | Olfm3+BMP4

B

Control: FLAG | Olfm3 | Olfm3+igG | BMP4 | Olfm3+anti-Olfm3 | Olfm3+BMP4

HUVEC: 15 min

C

Nuclear signal (mean intensity)

Control-FLAG | Olfm3+igG | Olfm3 | BMP4 | Olfm3+BMP4

D

pSMAD1/5/8

SMAD1

0 15 30 45 min

Olfm3 | Olfm3+BMP4

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