MET expression in melanoma correlates with a lymphangiogenic phenotype

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Melanomas contain high frequencies of tumorigenic cells and their tumorigenic capacity resides in several distinct subpopulations within melanoma. Since their metastatic potential is linked to their ability to recruit lymphatic vessels, we aimed at identifying lymphangiogenic subpopulations by comparative in vitro analysis of single cell clones derived from a melanoma of a single patient. Selected lymphangiogenic clones were then grafted into severe combined immunodeficient mice, where they induced lymphangiogenesis and metastasized into sentinel nodes, whereas non-lymphangiogenic clones from the same patient did not metastasize. Transcriptome analysis revealed high expression of vascular endothelial growth factor C (VEGF-C) and platelet derived growth factor C (PDGF-C) as well as of the met proto-oncogene (MET) and its targets to be associated with this lymphangiogenic phenotype. Screening of a set of independently isolated melanoma cell lines from other patients confirmed this association between expression of high levels of MET and of VEGF-C and PDGF-C. Hence, we provide a model to screen for the lymphangiogenic potential of tumor cells. We show that the lymphangiogenic potential is heterogeneously distributed among melanoma cells within one given tumor and is associated with activation of MET signaling.

INTRODUCTION

Melanoma lesions are composed of heterogeneous tumor cells (1–3). This phenotypic and genotypic heterogeneity is established by clonal evolution, by cancer stem cells or by a combination of both and is still a matter of debate (4,5). What has become clear is that a high proportion of single melanoma cells, isolated either from the primary tumor or from metastatic sites, are able to form tumors upon transplantation into non-obese diabetic/severe combined immunodeficient (NOD/SCID) interleukin-2 receptor gamma chain null (NSG) mice (6). What is not known until now is the contribution of heterogenic subpopulations derived from an individual tumor to the angiogenic process. Here, we introduce a working model on how to identify intratumor subpopulations and exemplify characteristics of lymphangiogenic melanoma subpopulations.

In contrast to blood angiogenesis, which is essential for tumor growth and is in part induced via hypoxia (7), lymphangiogenesis has been shown to be actively induced in only certain types of malignant tumors (8–12). Furthermore, intra- and peritumoral lymphangiogenesis has been proposed to be a prognostic factor for the induction of a metastatic niche, which subsequently leads to sentinel lymph node metastasis (13). Therefore, it is of paramount importance to identify signaling pathways activated in tumor cells that lead to secretion of lymphangiogenic factors. The most prominent human lymphangiogenic factors include vascular endothelial growth factor C (VEGF-C) and VEGF-D, which, after proteolytic processing, bind to VEGFR3 and VEGFR2 (14). In mice, deletion of VEGF-C leads to a complete lack of lymphatic endothelial cells (LECs), but in contrast, deletion of VEGF-D does not
affect the development of the lymphatic vasculature (15). Additionally, also other growth factors like: fibroblast growth factor 2, insulin-like growth factor (IGF-1 and IGF-2), angiopoietin (ANG-1 and ANG-2), placental growth factor (PlGF) and platelet derived growth factor B (PDGF-B) have been reported to be involved in the process of lymphangiogenesis (16–20).

A powerful tool for the identification of genes involved in organ-specific metastasis is the in vivo selection of tumor subpopulations (21). This method led to the discovery of gene profiles indicative for bone and lung metastasis during progression of breast cancer (22,23). Here, we modified this approach by using single cell clones generated from a single melanoma lesion as illustrated in Figure 1A. This enabled us to characterize subpopulations for their lymphangiogenic potential and allowed the transcriptomic analysis in a uniform genetic background, since all subpopulations were derived from the same donor. Hence, here we present a model system for the identification of pro-lymphangiogenic signals within heterotypic melanoma lesions. Using this system, we show that the met proto-oncogene (MET) up-regulation in melanoma is accompanied by up-regulation of VEGF-C and PDGF-C, lymphangiogenesis and metastasis.

RESULTS
Characterization of single cell clones from an individual melanoma lesion
The selection procedure applied is schematically shown (Fig. 1A). Initially, we harvested melanoma tissue from a
total of 19 patients. We succeeded in creating bulk cultures from six lesions. Here, we report on a bulk culture termed MCM1, which was derived from a 61-year-old male diagnosed with a nodular melanoma stage T2a, N0. At passage 3 of culturing, DNA was isolated and Affymetrix SNP6.0 array hybridizations were performed. Cultured MCM1 cells revealed overall identical copy number gains and losses when compared with the primary patient-derived lesion and were therefore used in this study (Fig. 1B and C). The complex mutation profile detected in both samples displays typical chromosomal aberrations frequently found in cutaneous melanoma, e.g. +6p, 6q- and -10 (24). Furthermore, sequence mutation analysis revealed MCM1 cells to be wild-type in NRAS exon 2 and heterozygous for the V600E BRAF mutation (data not shown). From the MCM1 bulk culture, a total of 17 single cell clones were generated, from which 11 could be subpassaged successfully (>30 passages). Passed cells were immunophenotyped and all except for MCM1Q were found positive for S100b (Fig. 1D) and all were negative for HMB45 and MelanA (data not shown).

Subclones show different lymphangiogenic potential in vitro and in vivo

To test for the lymphangiogenic potential of isolated subclones, we incubated primary human LECs with the conditioned growth medium derived from the respective melanoma subclones and assessed LEC growth and survival. Primary LECs were derived from foreskins and they uniformly expressed lymphendothelial markers CD31, podoplanin and PROX1 (Supplementary Material, Fig. S1). Incubation with supernatant from clone MCM1D lead to a significant increase in the amount of vital cells over a time period of 72 h, compared with the parental cell line MCM1 and other clones (Fig. 1E).

To analyze the lymphangiogenic potential of the primary culture (MCM1) and of eight corresponding subclones in vitro, cells were injected intradermally into flanks of at least four different CB.17 SCID mice per cell line. Three formed tumors (MCM1, MCM1D and MCM1G) following an observation period of 3 months after engraftment (Table 1). Labeling with a human-specific anti-vimentin antibody enabled us to visualize in vivo cell invasion and only in MCM1D melanoma cells were trapped within luminal structures, morphologically resembling lymphatics, distant from the primary lesion (Fig. 2A). In 1 out of 10 mice transplanted with MCM1D cells axillary, brachial and inguinal lymph nodes contained melanoma cells. These cells were isolated, expanded in culture and termed MCM1DLN. Upon re-transplantation, MCM1DLN formed tumors, which were S100b positive, but devoid of melanoma differentiation markers SOX10, p75NTR and nestin (Fig. 2B). MCM1DLN consistently metastasizes to sentinel lymph nodes and the lung in all the eight tested mice (Table 1). In MCM1 and MCM1G tumors, lymphatic vessels were rare. In contrast, MCM1D and MCM1DLN induced significant blood and lymphangiogenesis, as demonstrated by CD34 and Lyve1 immunohistochemistry (Fig. 3A–D) and by real-time polymerase chain reaction (PCR) analysis for Prox1 and Lyve1 mRNA (Fig. 3E).

| Table 1. Characterization of single cell clones from the MCM1 pool of parental melanoma cells |
|----------------|----------------|---------------|-------------|-------------|----------------|
| Cell line | Growth | In vitro | Lymph Angio. | LN Meta. | Lung Meta. |
| MCM1 | + | 10/10 | Neg. | 0/10 | 0/10 |
| MCM1A | + | 0/6 | – | – | – |
| MCM1B | + | 0/6 | – | – | – |
| MCM1C | – | – | – | – | – |
| MCM1D | + | 10/10 | Pos. | 1/10 | 0/10 |
| MCM1DLN | + | 8/8 | Pos. | 8/8 | 8/8 |
| MCM1E | – | – | – | – | – |
| MCM1F | – | – | – | – | – |
| MCM1G | + | 10/10 | Neg. | 0/10 | 0/10 |
| MCM1H | + | – | – | – | – |
| MCM1I | + | 0/4 | – | – | – |
| MCM1J | + | – | – | – | – |
| MCM1K | – | – | – | – | – |
| MCM1L | + | 0/4 | – | – | – |
| MCM1M | + | 0/4 | – | – | – |
| MCM1N | – | – | – | – | – |
| MCM1O | – | – | – | – | – |
| MCM1P | – | – | – | – | – |
| MCM1Q | + | 0/4 | – | – | – |

Growth in vitro: + denotes culturing for >30 passages. Growth in vivo: number of C.B. 17 SCID mice with tumors >300 mm3 3 months after inoculation per total number of mice tested. Lymph angio., lymphatic angiogenesis; neg., <10 Lyve1-positive vessels per mm2; pos., more than 10 Lyve1-positive vessels per mm2; LN, lymph node; meta., metastasis; numbers indicate number of C.B. 17 SCID mice, which harbored human vimentin-positive cells in proximal lymph nodes or lungs per total number of mice tested. –, not tested.

Transcriptome profiling of lymphangiogenic subpopulations

To identify the genes involved in the regulation of lymphangiogenesis, we performed a human transcriptome analysis. RNA was extracted from MCM1D-, MCM1DLN-, MCM1G- and MCM1-derived tumors after intradermal transplantation into C.B.17 SCID mice. Cluster dendrogram and principal component analysis separated lymphangiogenic from non-lymphangiogenic tumors (Supplementary Material, Fig. S2A and B). Gene set enrichment analysis containing the gene ontology term biological process, cellular component and molecular function identified 130 gene sets enriched in the MCM1D and MCM1DLN group (nominal P-value <1%) and 38 gene sets enriched in the MCM1 and MCM1G group (nominal P-value <1%) (25,26). As an example, the gene set ‘extracellular matrix’ and ‘neuronal differentiation’ are shown (Supplementary Material, Fig. S3A and B).

Screening the expression profiles for mRNA levels of lymphangiogenic factors identified significant up-regulation of VEGF-C and PDGF-C (Fig. 4A). In order to correlate the lymphangiogenic capacity of MCM1D and MCM1DLN clones with a distinct pathway activated in these cells when compared with MCM1 and MCM1G cells, we screened for expression of pathway-dependent gene sets. We identified a striking activation of genes comprising the ‘met-pathway’ gene set in MCM1D and DLN clones compared with MCM1 and MCM1G cells (normalized enrichment score of 1.881; nominal P-value < 0.001) (Fig. 4B). We therefore analyzed MET and p-MET protein expression and found elevated
expression of MET in MCM1D and MCM1DLN compared with MCM1 and MCM1G (Fig. 4C). Furthermore, stimulation of the MET receptor by its ligand, hepatocyte growth factor (HGF), induced tyrosine phosphorylation in MCM1DLN but not in MCM1 cells (as tested with polyclonal rabbit antibody, which recognizes phosphorylation at positions 1230, 1234 and 1235). Specificity of the HGF-induced phosphorylation was confirmed by blocking the phosphorylation with the MET-specific small inhibitor PHA-665752 (Fig. 4D).

**MET expression correlates with VEGF-C and PDGF-C**

In order to test if this correlation between an activated MET pathway and expression of angiogenic factors is a common feature of melanoma, we screened for MET protein expression in six different melanoma cell lines isolated from tumors of human patients. Three out of six melanoma lines (Fig. 5A) showed increased amount of MET protein expression and increased phosphorylation of MET in response to HGF, which could also be blocked by PHA-665752. We then performed quantitative real-time PCR, and the group of MET\textsuperscript{high} cells had a 100-fold higher expression of VEGF-C and PDGF-C when compared with the group of MET\textsuperscript{low} cells (Fig. 5B). Of note, stimulation with HGF for 2 and 6 h did not further enhance and PHA-665752 treatment for 12 and 24 h did not decrease VEGF-C and PDGF-C expression. Therefore, MET status is only an indicator for the lymphangiogenic potential of melanoma and is not directly regulating expression of these genes (as shown previously in squamous cell carcinomas) (27).

**DISCUSSION**

Here, we introduce a protocol to assess the intratumor heterogeneity of melanoma. We generated single cell-derived melanoma lines from an individual human melanoma lesion for in vitro and in vivo screening of lymphangiogenic properties. The majority of the derived subpopulations were unable to induce lymph-angiogenesis and to metastasize to sentinel nodes, but a single subclone and its metastatic derivative...
induced lymph-angiogenesis and metastasized. Transcriptome profiling identified activation of the MET pathway to be associated with this particular lymphangiogenic phenotype. The association of MET expression and enhanced expression of lymphangiogenic factors could also be shown in melanoma cell lines derived from independently isolated human tumors.

Intratumor heterogeneity has been observed at the morphological, functional and genomic level in almost all tumor types. We recently demonstrated that heterogeneity within the melanoma initiating cell population has the potential to give rise to genetically distinct primary and metastatic melanoma lesions (28). One way to identify and isolate functionally

Figure 3. In vivo lymphangiogenesis of MCM1 subclones. (A) Representative immunohistochemical stainings for CD34 and (B) Lyve1 induced by indicated melanoma cell lines, which were xenografted into C.B.17 SCID mice until tumors reaches a volume of 300 mm³. Scale bar indicates 50 μm. (C) Quantification of CD34-positive and (D) Lyve1-positive vessels in immunohistochemical samples (n = 8). (E) mRNA was isolated directly from xenografted melanomas and subjected to quantitative real-time PCR for the lymphatic markers Lyve1 and Prox1 (n = 5). GAPDH served as internal control. Data are shown as means ± SD analyzed by Student’s t-test. *P < 0.05; **P < 0.01 versus MCM1.
different tumor cells within a given tumor is the in vivo selection of highly aggressive tumor cells (21–23). If a bulk culture of human tumor cells is used for in vivo selection, subpopulations present at very low frequencies may get lost during tumor progression and their contribution to the tumor phenotype remains unknown. Ways to circumvent this problem include transplantation of single tumor cells into fully immunocompromised mice (6) or to expand single cell-derived clones from the same melanoma lesion prior to transplantation in vitro, as shown here. Single cell-derived clones have the advantage that a high amount of tumor cells can be used, which increases chances of cell survival at the site of transplantation.

Melanoma primarily metastasizes to the sentinel lymph node and the amount of lymphangiogenesis correlates with the risk of sentinel-node metastasis (8,9,29–32). Here, we demonstrate that the generation of single cell-derived melanoma clones is a suitable approach to screen for lymphangiogenic clones and thereby to identify cofounders, which confer this enhanced lymphangiogenic capacity. Indeed, MCM1D cells, which induced proliferation of lymphatic endothelium in vitro, expressed high amounts of lymphangiogenic factors such as PDGF-C (33,34) and VEGF-C (9,15).

Since all subpopulations have been established from a single donor, the genetic background of all melanoma lines is identical, which eliminates inter-individual differences and allows to focus on expression changes due to altered cellular functions. Our principal component and cluster dendrogram analysis of expression data highlights expression differences...
between lymphangiogenic and non-lymphangiogenic clones. Based on pathway analysis, we found MET targets overexpressed in lymphangiogenic clones. Importantly, this correlated with increased MET mRNA and protein expression in melanoma clones able to induce lymphangiogenesis (MCM1D and MCM1DLN) when compared with MCM1 and MCM1G cells. MET is a receptor tyrosine kinase responsible for mitogenic and invasive properties of tumor cells (35). The receptor is activated upon binding of its proteolytically activated ligand HGF (36). It is only moderately expressed in melanocytes and primary melanomas, but in contrast, metastatic melanomas show significant up-regulation of MET (37). Therefore, MET is thought to be critically involved in melanoma progression, but its contribution to the lymphangiogenic process in melanoma is not defined. Despite the lack of evidence in melanoma, in other tumors, like oral squamous cell carcinoma, ductal breast carcinoma in situ and primary colon cancer, intratumoral MET expression has recently been linked to lymphangiogenesis (27,38,39). Interestingly, it has been shown that intratumoral HGF expression did not correlate with elevated levels of MET (27). In our model system, we could not detect differing HGF levels between metastatic and non-metastatic tumors (data not shown). Still, in vitro, high MET expressing cells responded to HGF stimulation with strong MET phosphorylation, whereas MET low expressing cells showed minimal MET phosphorylation in response to HGF. Therefore, one could speculate that in our melanoma lines, the amount of MET and not the amount of HGF is regulating the MET pathway activation.

It has been shown that MET levels correlate with VEGF-A and VEGF-C expression in squamous cell and ductal breast carcinoma (27,38) and that inhibition of MET by the selective small molecule inhibitor PHA 665752 reduces tumor growth and angiogenesis in human lung cancer xenograft models (40,41). We found enhanced expression of VEGF-C and PDGF-C in lymphangiogenic clones. Since expression of these factors was not enhanced by HGF, nor was it reduced by blocking MET activation with PHA665752, we exclude a direct effect of MET on VEGF-C and PDGF-C expression levels. To analyze if MET is indirectly associated with up-regulation of VEGF-C and PDGF-C, we analyzed six additional and independently isolated melanoma cell lines for MET, VEGF-C, PDGF-C and VEGF-A and we found the same correlation, which indicates high MET expression correlated with high growth factor expression.

VEGF-C is well known to induce proliferation of LECs, but PDGF-C has so far not been described this context. PDGF-C is a potent mitogenic factor and also a chemoattractant for cancer-associated fibroblasts, for macrophages and for blood endothelial cells (33,42–44). PDGF-C binds to the PDGFR-α homodimer and PDGFRβ heterodimer, just like PDGF-A, which is strongly downregulated in our lymphangiogenic clones. The biologic relevance of PDGF-C for the direct induction of lymphangiogenesis still remains to be defined.

In conclusion, here we present an in vitro and in vivo screening model for the identification of subclones with distinct phenotypes within an individual tumor lesion. We identified a lymphangiogenic subpopulation, and this screening system could as well be used for identification of clones acting on other stromal cells such as fibroblasts or blood endothelium. Furthermore, by transcriptome analysis, we identified the MET pathway to be associated with the lymphangiogenic phenotype. Still, in our system, MET is not a direct regulator of VEGF-C and PDGF-C, two angiogenic factors highly overexpressed in our lymphangiogenic subpopulations. Therefore, expanding this screening system to multiple single cell-derived clones from several donors is needed to generate a signature for melanoma subpopulations able to induce lymphangiogenesis. This will require an adaption of the screening procedure for high throughput analysis of a larger patient population. Subsequently, this could result in the identification of a consensus expression profile predictive for the lymphangiogenic potential of melanoma lesions. For clinical translation, expression of this consensus marker profile has to be analyzed in human melanoma lesions. Results have to be correlated with lymphangiogenesis in primary lesions and with the status of sentinel nodes being positive or negative for metastatic cells. Future work may then clarify if therapeutic targeting of this lymphangiogenic subpopulation could prevent the onset of sentinel-node metastasis.
MATERIALS AND METHODS

Tissue sampling and generation of the MCM1 cell pool

The procedure was approved by the ethics committee of the Vienna Medical School (EK 1088/2009). DNA from the patient-derived melanoma lesion and from the thereof derived melanoma cell line termed MCM1 was isolated by a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Mutation analysis in exon 15 of BRAF and of exon 2 in NRAS was performed as previously described (28). Affymetrix GeneChip Human Mapping SNP6.0 chips were hybridized using the manufacturer’s protocol (Affymetrix, Santa Clara, CA, USA). Overall hybridization quality was estimated by the call rate index obtained from GeneChip Genotyping Analysis Software (birdseedv2 algorithm using default parameter settings) and results for whole genome karyoview were visualized using the Affymetrix Genotyping Console 3.0.1 with default settings. All raw data were submitted to Gene Expression Omnibus and is available as series record GSE35991.

Genotyping

The genotyping of the primary melanoma was performed according to the approval of the ethics committee of the Vienna Medical School (EK 1088/2009). DNA from the patient-derived melanoma lesion and from the thereof derived melanoma cell line termed MCM1 was isolated by a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Mutation analysis in exon 15 of BRAF and of exon 2 in NRAS was performed as previously described (28). Affymetrix GeneChip Human Mapping SNP6.0 chips were hybridized using the manufacturer’s protocol (Affymetrix, Santa Clara, CA, USA). Overall hybridization quality was estimated by the call rate index obtained from GeneChip Genotyping Analysis Software (birdseedv2 algorithm using default parameter settings) and results for whole genome karyoview were visualized using the Affymetrix Genotyping Console 3.0.1 with default settings. All raw data were submitted to Gene Expression Omnibus and is available as series record GSE35991.

Immunocytochemical phenotyping

Melanoma cells were grown in eight-well chamber slides (Nunc, Langenselbold, Germany) in the standard DMEM medium and fixed in aceton/methanol (1:1) solution for 10 min at −20°C. Then the cells were blocked in phosphate-buffered saline containing 0.1% bovine serum albumin for 20 min at room temperature. Afterwards, cells were incubated with antibodies for melanoma markers S100b, HMB45 and MelanA (all Dako, Glostrup, Denmark, 1:200) over night at 4°C and subsequently incubated with biotinylated secondary antibodies (Jackson Lab, West Grove, PA, USA) for 30 min at room temperature. Aminoethyl carbazole (AEC, Dako) was used to visualize the staining (20 min) and the slides were counterstained with hematoxylin.

In vitro lymph-endothelial viability assay

LECs were isolated and passaged as described previously (46). Conditioned media from MCM1-derived clones were generated by incubation of one million cells in 3 ml of serum-free EGM-2MV medium (Cambrex, Walkersville, MD, USA) for 24 h. This medium was used to stimulate 2000 lymphatic endothelium cells (LECs), which were seeded into a 96-well plate. LECs were incubated for 72 h and cell growth and survival were measured using a tetrazolium salt reduction assay (EZ4U-proliferation Kit, Biomedica, Vienna, Austria). After 3 h of incubation, the absorbance was measured at 450 nm.

SCID mouse model and histology

The study protocol was performed in accordance with national laws and guidelines and was approved by the Medical University of Vienna’s Institutional Review Board (GZ: 66.009/0025-II/10b/2008 and GZ: 66.009/0031-II/3b/2011). The grafting procedure was described previously (47). Six- to 8-week-old CB.17 SCID/SCID female mice (Charles River, L’Arbresle, France) were injected intradermally with the respective melanoma cell lines into the lower left flank. Tumor volume was monitored every 4 days by caliper measurements until tumor volume exceeded 300 mm³. Primary tumors were then excised and 14 days later mice were sacrificed. Primary tumors, axillary, brachial and inguinal lymphnodes as well as the whole lung were fixed in 7.5% formaldehyde and embedded in paraffin. Tissue was sectioned and used for immunohistochemistry, using modified citrate buffer, pH 6.1 (Dako), for 20 min at 121°C for antigen unmasking and 3% H₂O₂ for 15 min at room temperature to quench endogenous peroxidases. Metastasis to lymphnodes and lungs was detected in H&E stains and confirmed by staining with human-specific anti-vimentin antibody (Dako). Primary tumors were immunostained with antibodies detecting SOX10, p75NTR (both Santa Cruz Biotech, Santa Cruz, CA, USA) and nestin (Chemicon, Temecula, CA, USA). Microvesel density was determined by incubating with antibodies detecting CD34 (BD/Pharmingen, San Diego, CA, USA, 1:50) and Lyve1 (Acris, Herford, Germany, 1:200) and by counting the total amount of vessels per area. Counting was performed by two qualified independent investigators and is displayed as vessels/mm².

RNA isolation and transcriptome expression profiling

The RNeasy Mini Kit (Qiagen) was used for the isolation of total RNA from primary tumors, which were intradermally grafted to C.B. 17 SCID mice and reached 300 mm³ of volume. RNA from 16 primary xenografted tumors derived from the four melanoma cell lines MCM1, MCM1G, MCM1D and MCM1DLN was isolated according to the standard protocols. An Agilent Bioanalyser (Agilent Technologies, Santa Clara CA, USA) was used to confirm the quality and integrity of the isolated samples.

Four samples per group were used for whole-transcript expression profiling, using GeneChip® Human Gene 1.0 ST Arrays (Affymetrix). The R (http://www.R-project.org/ Bioconductor Suite has been used for all subsequent analyses of the microarray data. RNA (48) was used for normalization and limma (49) for inferring differential expression between groups. All raw data were submitted to Gene Expression Omnibus and is available as series record GSE36035. Hierarchical clustering was performed using Pearson’s correlation coefficient as distance measure and the average linkage method to cluster genes and samples, respectively.
GSEA (25,26) was conducted using the gene set enrichment analysis offered by the Broad Institute. C2CP and C5 were used as gene set universe. Permutation type was set to gene set.

Western blotting
MET was stimulated by incubation with 40 ng/ml recombinant human HGF (R&D Systems, Minneapolis, MN, USA) and the stimulation was inhibited by an addition of 12.5 mM of the small molecule inhibitor PHA-665752 (Pfizer, La Jolla, CA, USA). Cells were lysed in RIPA buffer (Tris–HCl: 50 mM, pH 7.4, NP-40: 1%, Triton X-100: 1%, NaCl: 150 mM, ethylenediamine tetraacetic acid: 1 mM), 1% protease inhibitor and phosphatase inhibitor cocktail 2 and 3 (all Sigma-Aldrich, St Louis, MO, USA).

Ten nanograms of the lysates were loaded on 7% sodium dodecyl sulfate polyacrylamide gels. Primary antibodies for MET (sc-10, Santa Cruz Biotech), phosphorylated MET (pYpYpY1230/1234/1235) (44–888G, Invitrogen, Grand Island, NY, USA) and phosphatase inhibitor cocktail 2 and 3 (all Sigma-Aldrich, St Louis, MO, USA). The secondary antibodies used for western blotting were horseradish peroxidase conjugated (BioRad, Hercules, CA, USA). Signals were detected using the enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ, USA).

Real-time PCR
cDNA was synthesized from 5 µg total RNA, derived from mouse grafted melanoma cell lines, using the First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany) according to the standard protocol for real-time PCR purposes. In short, 2 µl random hexamer primer was mixed with the RNA and heated to 65°C to break potential secondary structures. The mixed reagents were then kept at 37°C for 1 h before the synthesis reaction was terminated by heating to 70°C for 5 min.

TagMan probes (Applied Biosystems, Carlsbad, CA, USA) were used for real-time PCR measurements performed in StepOne-Plus Systems (Applied Biosystems, Carlsbad, CA, USA). Primers used in this study (all from Applied Biosystems): glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), VEGF-A (Hs00173626_m1), VEGF-C (Hs00153458_m1), PDGF-C (Hs00211916_m1), beta-2 microglobulin (B2M) (Mm00437762_m1), Lyve1 (Mm00475056_m1) and Prox1 (Mm00435969_m1).

Excel was used for further data analysis. GAPDH was used for normalization of human targets and B2M as normalization for mouse targets. Student’s t-test was performed to compare fold changes in the xenografted tumors or in the melanoma cell lines.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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