VEGFR1-activity-independent metastasis formation

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Molecules such as vascular endothelial growth factor (VEGF) or placental growth factor—critical regulators of tumour angiogenesis—are also thought to mobilize into blood circulation bone marrow-derived cells (BMDCs)[1], which may subsequently be recruited to tumours and facilitate tumour growth and metastasis[2,3]. A study[4] has suggested that BMDCs form 'metastatic niches' in lungs before arrival of cancer cells, and showed that pharmacological inhibition of VEGF receptor 1 (VEGFR1, also known as Flt1)—cognate receptor for VEGF and placental growth factor—prevented BMDC infiltration in lungs and 'metastatic niche' formation. Here we report that blockade of VEGFR1 activity does not affect the rate of spontaneous metastasis formation in a clinically relevant and widely used preclinical model[5–8]. Therefore, alternative pathways probably mediate the priming of tissues for metastasis.

We assessed the role of VEGFR1 activity in spontaneous formation of macroscopic metastasis after pharmacological inhibition of VEGFR1 (with MF1-blocking antibody). We generated chimaeric C57BL mice in which BMDCs express green fluorescent protein (GFP) after lethal irradiation and restorative bone marrow transplantation (BMT) with cells from Actb-GFP/C57BL mice (BMT-Actb-GFP/C57BL mice). After bone marrow reconstitution, we subcutaneously implanted Lewis lung carcinoma (LLC1/LL2, a BMDC-rich tumour) or B16 melanoma (a tumour with significantly fewer BMDCs). Continuous treatment with MF1 did not delay primary tumour growth compared to non-specific IgG. Moreover, MF1 treatment did not reduce BMDC infiltration in LLC1 or B16 tumours (Fig. 1). We resected these tumours when they had grown to 1 cm in diameter (after 15–17 days) and metastatic foci had already formed in the lungs[5,6]. As no macroscopic metastases were detectable at the time of primary tumour removal, we measured the number of BMDCs in the lungs of BMT-Actb-GFP/C57BL mice before and after the formation of macroscopic metastases. BMDC infiltration at the time of resection (day 0) and at day 10 was relatively small but readily detectable and comparable with overall BMDC accumulation in tumour-free BMT-Actb-GFP/C57BL mice in both tumour models (Fig. 1).

This suggests a key role for activated pulmonary alveolar macrophages—BMDCs that reside in the normal lung in comparable numbers in tumour-free non-BMT C57BL mice—as opposed to *de novo* BMDC recruitment before spontaneous metastasis. When most mice spontaneously developed macroscopic metastases (day 14), we detected a significant increase in *de novo* BMDC accumulation inside the LLC1 metastatic nodules and in the peri-tumour lung tissue, but not in B16 metastases, akin to BMDC incorporation in the primary tumours in these models (Fig. 1). VEGFR1 blockade with MF1 significantly reduced BMDC accumulation in LLC1 metastases. Surprisingly, VEGFR1 blockade did not decrease the occurrence, number, size or overall burden of spontaneous lung metastases on day 14 after LLC1 or B16 resection compared to control-treated mice (Table 1).

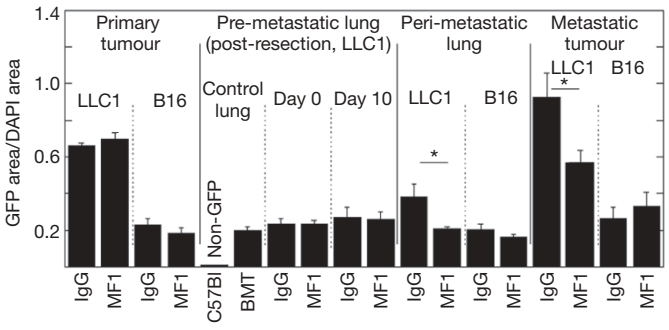

Figure 1 | Effect of blocking VEGFR1 activity on BMDC accumulation in tumours and metastatic lung tissues. The number of BMDCs was calculated as ratio of GFP-surface area to DAPI-surface area (±s.e.m.). DAPI was used to stain the nuclei of all cells ($n = 6$–8 mice per group). *$P < 0.05$.

Table 1 | Quantification of spontaneous metastasis formation after blockade of VEGFR1 activity

Tumour / model		BMT-Actb-GFP/C57BL		C57BL	*flt1*$^{TK-/-}$/C57BL
		MF1	IgG		
LLC1	No. of mice	9/9 (100%)	8/8 (100%)	6/8 (75%)	12/13 (92%)
LLC1	No. of nodules	13 ± 4	15 ± 6	10 ± 4	12 ± 4
P value		0.41		0.32	
B16	No. of mice	9/12 (75%)	9/13 (69%)	6/8 (75%)	7/14 (50%)
B16	No. of nodules	9 ± 4	6 ± 2	2 ± 1	3 ± 2
P value		0.21		0.32	

Data are shown as number of mice with spontaneous lung metastases on day 14 and as number of macroscopic lung tumour nodules per mouse (mean ± s.e.m.). *P* values were calculated with a Student's *t*-test and showed no significant difference. In addition, rank comparisons with the Mann–Whitney *U*-test showed no significant differences

Previous studies in mice genetically deficient in VEGFR1-tyrosine kinase domain (*flt1*$^{TK-/-}$ mice) have shown that MMP-9 is induced by VEGFR1 signalling in lung stromal cells, which facilitates metastatic tumour growth in experimental metastasis models (that is, after intravenous infusion of cancer cells)[9]. When tested in *flt1*$^{TK-/-}$/C57BL mice, LLC1 growth was similar and B16 growth was slightly delayed compared to C57BL mice. However, when evaluated at day 14, the number of mice with spontaneous macroscopic lung metastases or the number of metastatic nodules was not significantly different (Table 1). CD11b (Mac1) immunostaining in normal and metastatic lungs of these mice showed comparable myeloid BMDC infiltration in *flt1*$^{TK-/-}$/C57BL and C57BL mice. Finally, since the extracellular domain of VEGFR1 is present on cells in *flt1*$^{TK-/-}$/C57BL mice, we measured by flow cytometry the number of VEGFR1$^+$ cells in circulating blood and in the tumour tissue. We detected no significant difference in circulating VEGFR1$^+$ cells in LLC1- or B16-burdened mice, or in tissue-resident VEGFR1$^+$ cells in normal lung or LLC1 tumours, and only marginal differences in B16 tumours[10].

In conclusion, VEGFR1 modulates BMDC infiltration and primary/metastatic tumour growth in some models, consistent with previous reports[11]. However, pharmacological blockade or genetic deficiency in intracellular VEGFR1-TK domain neither eradicated nor significantly altered pre-metastatic BMDC infiltration or early spontaneous metastasis formation in lungs in these models.

METHODS

Flt1$^{TK-/-}$/C57BL (re-derived from *flt1*$^{TK-/-}$ mice[10,12], kindly provided by M. Shibuya, Univ. Tokyo) and Actb-GFP/C57BL mice (Jackson Labs), C57BL mouse-syngeneic LLC1 lung carcinoma (CRL-1642) and B16 melanoma cell lines (CRL-6323)(both ATCC), the BMT and spontaneous metastasis formation models, and confocal microscopy and flow cytometric analyses have been previously described[5,10,12]. We used Matlab software for quantification. Rat anti-VEGFR1 monoclonal antibody (MF1, a gift from ImClone Systems) or IgG (Jackson Labs) was administered intraperitoneally (20 or 40 mg kg^{-1}) to tumour-bearing mice three times per week[13]. We used Alexa-Fluor-647 (Molecular Probes)-labelled MF1 for flow cytometry. All other antibodies were purchased from BD-Pharmingen.

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1. Rafii, S., Lyden, D., Benezra, R., Hattori, K. & Heissig, B. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nature Rev. Cancer* **2,** 826–835 (2002).
2. de Visser, K. E., Eichten, A. & Coussens, L. M. Paradoxical roles of the immune system during cancer development. *Nature Rev. Cancer* **6,** 24–37 (2006).
3. Pollard, J. W. *Nature Rev. Cancer* Tumour-educated macrophages promote tumour progression and metastasis. **4,** 71–78 (2004).
4. Kaplan, R. N. *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438,** 820–827 (2005).
5. Duda, D. G. *et al.* Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors. *Blood* **107,** 2774–2776 (2006).
6. Gao, D. *et al.* Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. *Science* **319,** 195–198 (2008).
7. Ohtaki, T. *et al.* Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* **411,** 613–617 (2001).
8. Padua, D. *et al.* TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133,** 66–77 (2008).
9. Hiratsuka, S. *et al.* MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* **2,** 289–300 (2002).
10. Dawson, M. R., Duda, D. G., Chae, S.-S., Fukumura, D. & Jain, R. K. VEGFR1 activity modulates myeloid cell infiltration in growing lung metastases but is not required for spontaneous metastasis formation. *PLoS One* doi:10.1371/journal.pone.0006525 (2009).
11. Fischer, C. *et al.* Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* **131,** 463–475 (2007).
12. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. & Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl Acad. Sci. USA* **95,** 9349–9354 (1998).
13. Wu, Y. *et al.* Anti-vascular endothelial growth factor receptor-1 antagonist antibody as a therapeutic agent for cancer. *Clin. Cancer Res.* **12,** 6573–6584 (2006).

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Kaplan et al. *reply*

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Commenting on ref. 1, Dawson *et al.*[2] claim that metastasis formation is independent of VEGFR1 activity, contradicting work by us and many others, including the original description of *flt1*$^{TK-/-}$ (VEGFR1-TK$^{-/-}$) mice in the metastatic setting[3]. Contrasting the findings by Dawson *et al.*[2], here we show that VEGFR1 knockdown in myelomonocytic cells eradicates micro- and macrometastases in a non-amputation/resection tumour model.

Hiratsuka *et al.*[3] originally reported that *flt1*$^{TK-/-}$ mice have impaired metastatic progression due to a significant reduction in MMP9^{+}CD11b^{+} (Mac1^{+}) cell infiltration in pre-metastatic lungs following inoculation with LLC or B16 tumour cells. Moreover, several reports demonstrate that inhibition of VEGFR1 by anti-VEGFR1 peptide blocks micro- and macrometastasis, while overexpression of placental growth factor (PlGF), which signals exclusively through

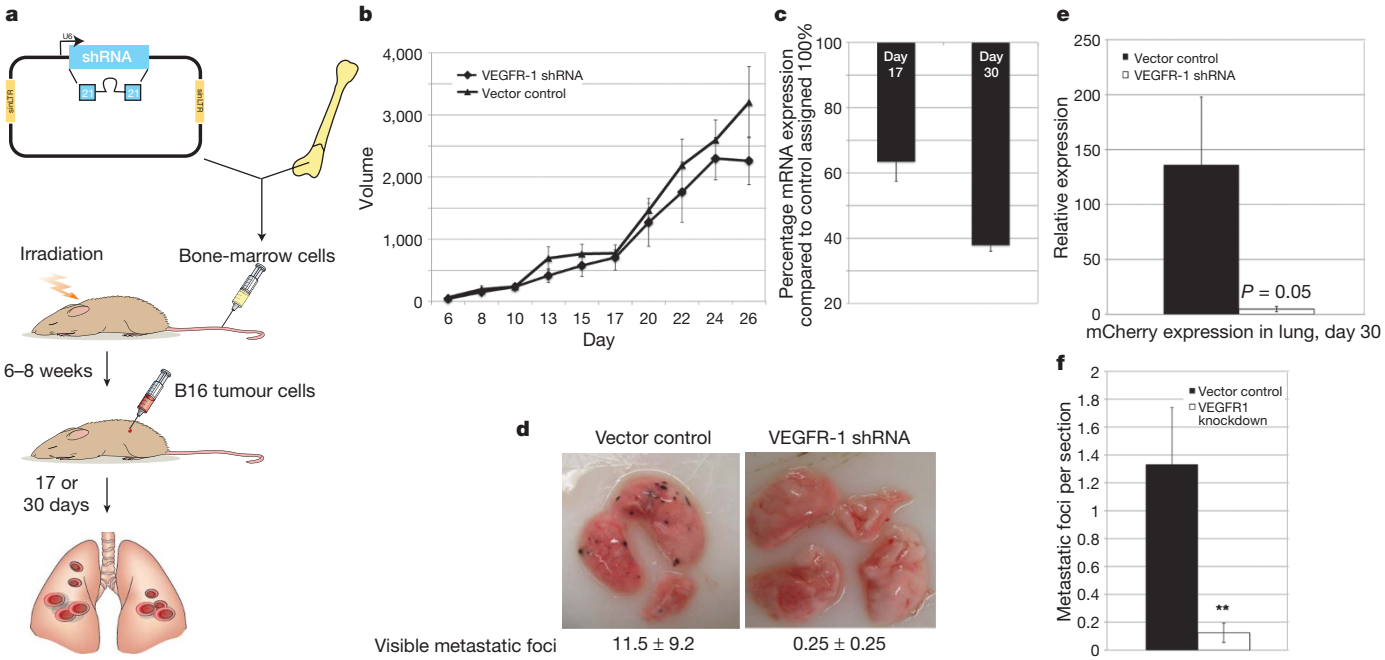

Figure 1 | Knockdown of VEGFR1 in the bone marrow inhibits metastatic progression. **a**, Schematic showing experimental design. Lineage-depleted bone marrow cells were transduced with a lentiviral vector driving ubiquitous expression of VEGFR1 shRNA. Transduced cells were used for bone marrow transplants. Six to eight weeks after transplantation, mice were given subcutaneous injections of mCherry-B16 tumour cells (day 0) and lungs were analysed at day 17 and day 30. **b**, Time course indicating primary tumour volume (v) throughout the experiment; v was calculated using $v = (lwh)/(2\pi/3)$, where l is length, w is width and h is height; $n = 7$ for days 0–17; $n = 4$ for days 18–26. **c**, Quantitative PCR analysis of VEGFR1 expression in peripheral blood of VEGFR1 shRNA mice at days 17 and 30. Data are represented as percentage of mRNA expression compared to average of vector control mice ($n = 4$). **d**, Images illustrating visible metastatic foci in lungs at day 30 after B16 melanoma tumour injection. Data are mean visible metastatic foci per lung ± s.e.m. ($n = 4$). **e**, Quantitative PCR analysis of tumour-derived mCherry expression in lungs at day 30 ($n = 4$). **f**, Quantification of micrometastatic lesions (defined by >20 tumour cells) visible per lung section at day 30. Six sections per mouse ($n = 4$). Data are mean ± s.e.m. $^{**}P < 0.01$, Student's t-test.

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VEGFR1, increases metastatic spread[4,5]. Thus, caution is necessary when drawing conclusions from the findings of Dawson *et al.*[2] regarding the role of VEGFR1 in metastasis.

In deviation from metastasis models used by us and others to document the existence of the pre-metastatic niche[1,3,6–9], Dawson *et al.*[2] use a model whereby primary tumours were implanted in the hind limb and resected by amputation of the leg, thus producing a sizeable wound before examining metastatic progression in the lung. Resection of the primary tumour at this early time point eliminates several tumour-derived paracrine factors, including VEGF-A, TNF-α, TGF-β, S100A8-SAA3[6,7], lysyl oxidase[8], IL6 and versican[9], factors that have been shown to recruit myeloid cells to the pre-metastatic niche and promote metastatic progression. Thus, it is plausible that removal of the primary tumour and generation of a traumatic wound caused a significant modulation of systemic metastatic niche supportive factors, resulting in disabled recruitment and/or function of VEGFR1-expressing bone marrow-derived cells (BMDCs), at metastatic sites. Although their experimental design may provide a relevant model for the effects of primary surgical resection on metastasis, the findings by Dawson *et al.*[2] cannot exclude VEGFR1 function in metastasis in non-tumour resection/amputation models.

The methodology used to evaluate metastasis in the lung by Dawson *et al.*[2] was insufficient and further confounds interpretation of their findings. Metastasis was evaluated grossly by counting metastatic nodules (Fig. 1; ref. 2). More quantitative measures may yield differing results, given both the risk of underestimating tumour burden by gross examination and the role VEGFR1 may play in both micro- and macrometastatic disease.

Many studies have confirmed that infiltration of myeloid cells is essential for metastasis[10,11]. Current data suggest that many pro-angiogenic myeloid cells express VEGFR1[12,13]. Furthermore, these studies highlight inhibition of VEGFR1 to target metastasis and PlGF-dependent tumour models in which VEGFR1 signalling is specifically targeted[10,14]. In these models, there is a profound defect in recruitment of pro-angiogenic and pre-metastatic VEGFR1+ myeloid cells, resulting in a reduction of metastasis.

An additional important consideration is that a complete absence of VEGFR1-mediated signalling in the *flt1*$^{TK-/-}$ mouse model used in this study has not been proven. Given the promiscuity of tyrosine kinase receptors, it is likely that the extracellular domain of the kinase retains some signalling activity through cross-talk with other activated tyrosine kinases or integrins. This residual activity is also supported by the finding that *flt1*$^{TK-/-}$ mice are viable, while mice lacking the full-length VEGFR1 die *in utero*[15]. In addition, VEGFR1 is expressed in many different cell types, including haematopoietic, endothelial and stromal cells, and non-cell-specific deletion of VEGFR1 in this model does not specifically address the role of its expression in BMDCs.

To address this question and expand on the brief functional analyses of VEGFR1 inhibition presented in ref. 1, we used lentiviral vectors to knockdown VEGFR1 activity specifically in BMDCs (Fig. 1a). Consistent with other reports[1,3,7], inhibition of VEGFR1 by short hairpin (sh)RNA did not significantly alter primary B16 melanoma tumour growth (Fig. 1b, c). However, analysis of the lungs 30 days post-implantation revealed a pronounced reduction in the number of metastatic lesions and total tumour burden in mice with bone marrow transduced with VEGFR1 shRNA as compared to controls (Fig. 1d–f). This observation indicates that VEGFR1 expression by BMDCs is necessary for metastatic tumour progression. Thus, a genetic approach using a non-amputation/resection model can serve to resolve the controversy surrounding the function of VEGFR1 in mediating metastasis. We hope that the field of metastatic initiation continues to move forward, given its profound impact in the clinical setting. Moreover, anti-PlGF/VEGFR1 agents may provide novel means to block micrometastasis in an adjuvant setting and inhibit metastatic progression.

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1. Kaplan, R. N. *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438,** 820–827 (2005).
2. Dawson, M. R., Duda, D. G., Fukumura, D. & Jain, R. K. VEGFR1-activity-independent metastasis formation. *Nature* **461,** doi:10.1038/nature08254 (2009).
3. Hiratsuka, S. *et al.* MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* **2,** 289–300 (2002).
4. Marcellini, M. *et al.* Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor. *Am. J. Pathol.* **169,** 643–654 (2006).
5. Taylor, A. P. & Goldenberg, D. M. Role of placenta growth factor in malignancy and evidence that an antagonistic PlGF/Flt-1 peptide inhibits the growth and metastasis of human breast cancer xenografts. *Mol. Cancer Ther.* **6,** 524–531 (2007).
6. Hiratsuka, S. *et al.* The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a premetastatic phase. *Nature Cell Biol.* **10,** 1349–1355 (2008).
7. Hiratsuka, S., Watanabe, A., Aburatani, H. & Maru, Y. Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nature Cell Biol.* **8,** 1369–1375 (2006).
8. Erler, J. T. *et al.* Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* **15,** 35–44 (2009).
9. Kim, S. *et al.* Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature* **457,** 102–106 (2009).
10. Fischer, C. *et al.* Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* **131,** 463–475 (2007).
11. Shojaei, F. *et al.* Bv8 regulates myeloid-cell-dependent tumour angiogenesis. *Nature* **450,** 825–831 (2007).
12. Du, R. *et al.* HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* **13,** 206–220 (2008).
13. Lin, E. Y. *et al.* VEGF restores delayed tumor progression in tumors depleted of macrophages. *Mol. Oncol.* **1,** 288–302 (2007).
14. Luttun, A. *et al.* Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nature Med.* **8,** 831–840 (2002).
15. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. & Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl Acad. Sci. USA* **95,** 9349–9354 (1998).

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