Angiopoietin-1 Overexpression Modulates Vascular Endothelium to Facilitate Tumor Cell Dissemination and Metastasis Establishment

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Abstract

The angiopoietin-1 (Ang1)/Tie2 signaling pathway is known to play an important role in the regulation of vascular maturation and maintenance of vessel integrity. In this study, we have investigated the effect of systemic Tie2 activation or inhibition on tumor growth and metastasis. We found that treatment with Ang1 delivered via an adenoviral vector promoted s.c. implanted tumor metastasis to the lungs. Ang1 treatment did not significantly increase vascular density in the tumors but induced enlargement of blood vessels in both the tumor and normal tissues, which increased tumor cell dissemination into the blood circulation. Ang1 also enhanced the formation of metastatic foci in the lungs when tumor cells were injected into the circulation via the tail vein. The effect of Ang1 on metastasis was validated by a simultaneous treatment with a soluble form of Tie2 (sTie2), which led to the suppression of Ang1-induced increase of tumor metastasis. Furthermore, using a highly metastatic tumor model, we confirmed that systemic treatment with sTie2 suppressed tumor metastasis to the lungs and lymph nodes, whereas tumor-associated angiogenesis and lymphangiogenesis were not significantly affected. This suggests that the Ang1/Tie2 signals contribute to tumor progression by increasing vascular entry and exit of tumor cells to facilitate tumor dissemination and establishment of metastases. [Cancer Res 2009;69(11): 4656–64]

Introduction

It is well known that tumor metastases, rather than the primary tumor, are the main cause of lethality for human patients. Tumor cells spread mainly via two pathways, the blood and lymphatic vessels. Tumor-associated angiogenesis is required not only for tumor growth but also for tumor metastasis to distant organs (1–3), and tumor-associated lymphangiogenesis promotes tumor spread via the lymphatic vessels (4–7). Furthermore, tumor cells and/or tumor-infiltrating stromal cells secrete a variety of growth factors such as vascular endothelial growth factors (VEGF) and angiopoietins, which are actively involved in the regulation of tumor angiogenesis and lymphangiogenesis (3, 8–10).

Angiopoietins are a family of four growth factors, which have opposing actions in blood vascular endothelial cells. Angiopoietin-1 (Ang1) and Ang4 stimulate Tie2 phosphorylation, whereas Ang2 is a context-dependent inhibitor/activator of Tie2 (11–13). Genetic analyses have revealed that Tie2-mediated signal transduction is important for the survival of vascular endothelium and angiogenic sprouting (12, 14, 15). Deletion of Ang1 results in a similar phenotype to that of Tie2 knockout (16), whereas mice deficient of Ang2 display defects in lymphatic vessels. This phenotype could be rescued in knock-in mice, wherein a cDNA encoding Ang1 was placed in the *Ang2* locus (17). Ang1 has also been shown to induce lymphatic sprouting and hyperplasia (18, 19). These results indicate that the angiopoietins regulate lymphatic vessel development.

Tie2 and Ang2 are up-regulated in tumor vessels (9). A soluble extracellular domain of Tie2 (sTie2) inhibited tumor angiogenesis and growth when applied locally to the tumor implantation site, and when delivered via adenoviral vectors, it inhibited primary tumor growth and metastasis at distant sites (20–22). However, it is not clear if the sTie2 target involved is Ang1 or Ang2, or both. Studies using ectopic expression of angiopoietins by tumor cells have yielded contradictory results about their role in tumor growth and metastasis. In one study, overexpression of Ang2 was shown to inhibit tumor metastasis formation (23), whereas in another, Ang2 blocking antibody or peptide-Fc fusion protein inhibited tumor angiogenesis and growth (24). In addition, Ang1 overexpression in human breast cancer or squamous cell carcinoma cells inhibited tumor growth (25, 26), but its overexpression in cervical cancer cells promoted tumor angiogenesis (27).

To clarify the role of Ang1/Tie2 signaling in tumor metastasis, we overexpressed Ang1 or sTie2 systemically via an adenoviral vector in tumor-bearing mice. We show that systemic treatment with Ang1 promotes tumor metastasis, whereas sTie2 suppresses lung and lymphatic metastasis, without significant changes in tumor-associated blood or lymphatic vessel density or tumor growth. Instead, we find that Ang1 facilitates tumor cell entry into the circulation and exit from the blood vessels to the lung. This suggests a novel role of the Ang1/Tie2 pathway in tumor progression by regulating vascular function to facilitate tumor dissemination and metastasis establishment.

Materials and Methods

Tumor cell lines. LNM35/Luc and LNM35/GFP tumor cells were established and maintained as previously described (28, 29). Luciferase-tagged NCI-H460 and MDA-MB435 tumor cells were established by transducing cells with recombinant lentiviral vectors expressing firefly

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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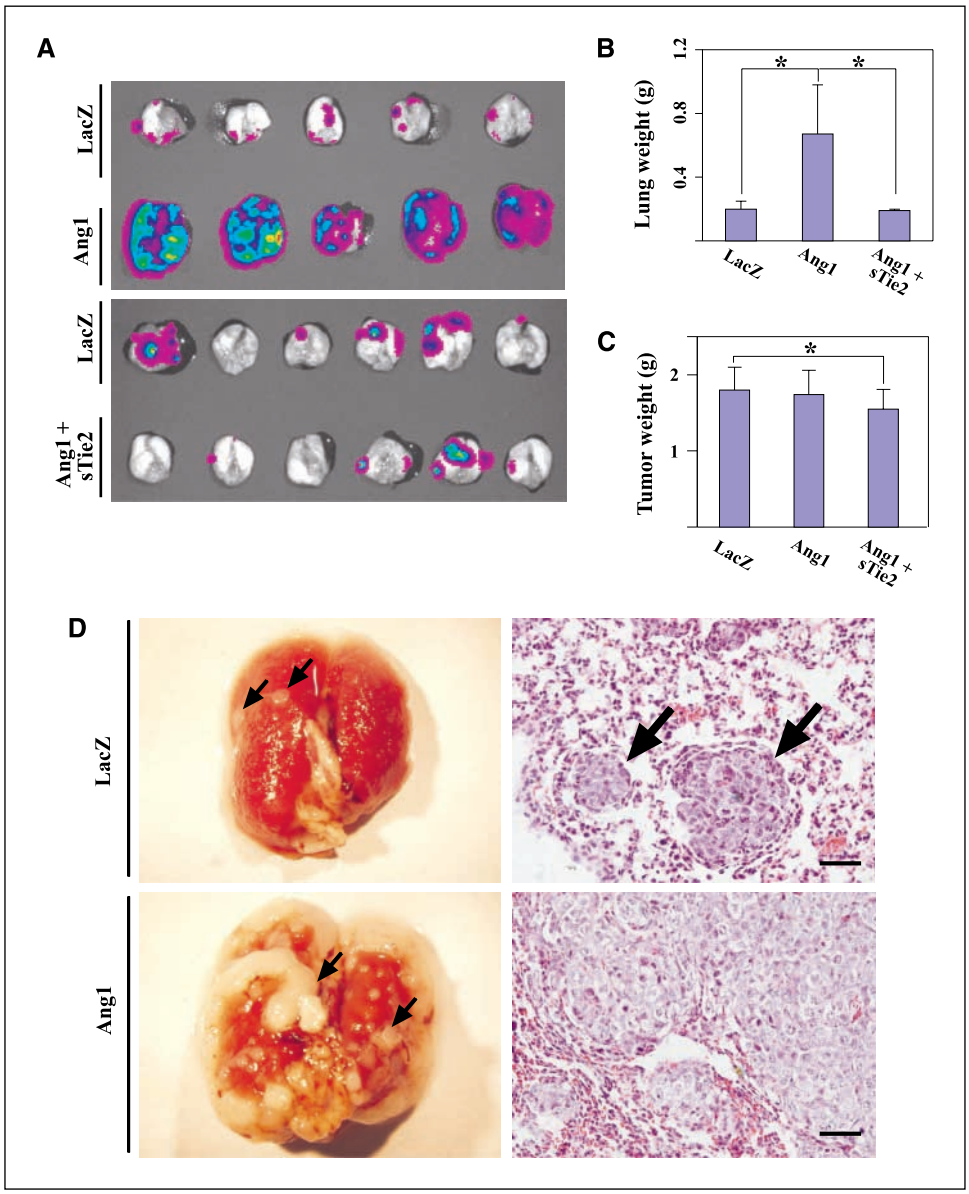

Figure 1. Systemic treatment with Ang1 promotes lung metastasis. *A,* representative bioluminescent images of lungs with metastasis from the mice treated with AdLacZ, AdAng1, or AdAng1 + AdsTie2. *B,* quantification of lung weight (LacZ versus Ang1: $P < 0.0001$; Ang1 versus Ang1 + sTie2: $P = 0.0004$). *C,* tumor weight at week 3 from the treated and control groups. *D,* lungs with metastatic lesions and H&E staining of the lung sections with metastatic nodules (*arrows*). Note that the lungs from the Ang1-treated mice are full of metastatic foci. *, $P < 0.05$. *Bar,* 50 μm.

luciferase, and tumor cells with stable expression of luciferase were selected by limiting dilution (30).

***In vivo* delivery of recombinant adenovirus and protein analysis.** Recombinant adenoviruses expressing sTie2 (20), Ang1 (18), COMP-Ang1 (31), or β-galactosidase (AdLacZ; ref. 32) were administered via the tail vein (0.5–1.0e+9 plaque-forming units per mouse). Blood was collected 1 wk after the treatment, and the serum concentration of recombinant proteins was determined by ELISA as previously described (20). To examine Tie2 activation or inhibition in lung by the treatment, mice were sacrificed 5 d after the adenoviral administration and lung tissues were collected. Tissues were homogenized and Tie2 phosphorylation was analyzed as previously described (33). Results shown in Supplementary Fig. S1 validated the *in vivo* agonistic or antagonistic activity of Ang1 or sTie2, respectively.

Xenotransplantation, tumor excision, and analysis. The study was approved by the Committee for Animal Experiments of the District of Southern Finland, and all animal experiments were done in accordance with the institutional guidelines of Model Animal Research Center of Nanjing University. Tumor implantation into severe combined immunodeficient mice and treatment with the recombinant adenoviruses were done as described previously (28, 34). Primary tumors were excised 3 wk after the tumor implantation. Mice were allowed to recover and analyzed within 5 wk. Internal organs including the lungs and axillary lymph nodes were collected, weighed, and processed for histology.

In separate experiments, recombinant adenoviruses were administered 2 d before i.v. injection of tumor cells (3.0e+6 cells in 200 μL). Mice were analyzed for metastasis formation using a fluorescence microscope or *in vivo* bioluminescance imaging.

***In vivo* imaging of tumor metastasis and quantification of bioluminescence signal.** *In vivo* imaging was done using the IVIS Imaging System (Xenogen) or PhotonImager (BioSpace) as previously described (28). After the imaging, the animals were euthanized, and organs of interest were collected and imaged *ex vivo*. A region of interest was manually selected, kept constant for all samples, and quantified as photons per second or counts per minute using the manufacturer's imaging software.

Luciferase activity assay. Luciferase activity in blood was measured by following the manufacturer's instruction (Promega). Briefly, 50 μL of blood were collected in an EDTA-containing tube. After lysing RBC, the remaining cells were collected by centrifugation and then lysed in 20 μL of lysis buffer. Ten microliters of cell lysate were mixed with 50 μL of the Luciferase Assay Reagent for light measurement in a TURNER BioSystem 20/20^n Luminometer [in relative light units (RLU)]. For measurement of luciferase activity in tissues, tissue lysate was used for analysis as above.

Immunohistochemical staining. Paraffin or frozen sections (6–10 μm) were immunostained with monoclonal antibodies against platelet/endothelial cell adhesion molecule 1 (PECAM-1; PharMingen) or LYVE-1 (28, 35). Quantification of blood vessel number and area was done using Image-Pro Plus (v5.1.2, MediaCybemetics).

Reverse transcription-PCR. Total RNA from cultured tumor cells or tumor tissues was extracted using TRIzol (Invitrogen). Similar amounts of RNA from each sample were used for reverse transcription (Invitrogen) and amplification. Specific primer pairs are as follows: human Ang1 (hAng1), accagtcagaggcagtacatgc (for) and gagactcttgtgaactcaaacgg (rev); mouse Ang1 (mAng1), accagtcagaggcagtacatgc (for) and gtcaatgagaatgttaactgcctg (rev); human Ang2 (hAng2), agatcaaggcctactgtgacatg (for) and ggacatatgggtatttacacagtg (rev); and mouse Ang2 (mAng2), agatcaaggcctactgtgacatg (for) and cttctccagatgataacctgtgc (rev). RNA without reverse transcription was used as a negative control. cDNA synthesis was monitored by PCR for β-actin (for, agcacagagcctcgcctttgccga; rev, gccaatggtgatgacctggccgtca).

Statistical analysis. Statistical analysis was done with unpaired *t* test or Fisher's exact test. All statistical tests were two-tailed.

Results

Systemic treatment with Ang1 promotes tumor metastasis to the lungs. To study the effect of Ang1 on tumor progression, we treated mice systemically with Ang1 or COMP-Ang1 delivered via an adenoviral vector 1 day after s.c. implantation of LNM35/Luc, and AdLacZ was used as control. The serum level of COMP-Ang1 1 week after the treatment was 1.65 ± 0.11 μg/mL (mean ± SD, $n = 3$). Treatment with AdAng1 increased Tie2 phosphorylation in the lungs (Supplementary Fig. S1), which is consistent with the previous observation (33). Both Ang1 and COMP-Ang1 increased tumor metastasis. Shown in Fig. 1*A* are representative images of lungs

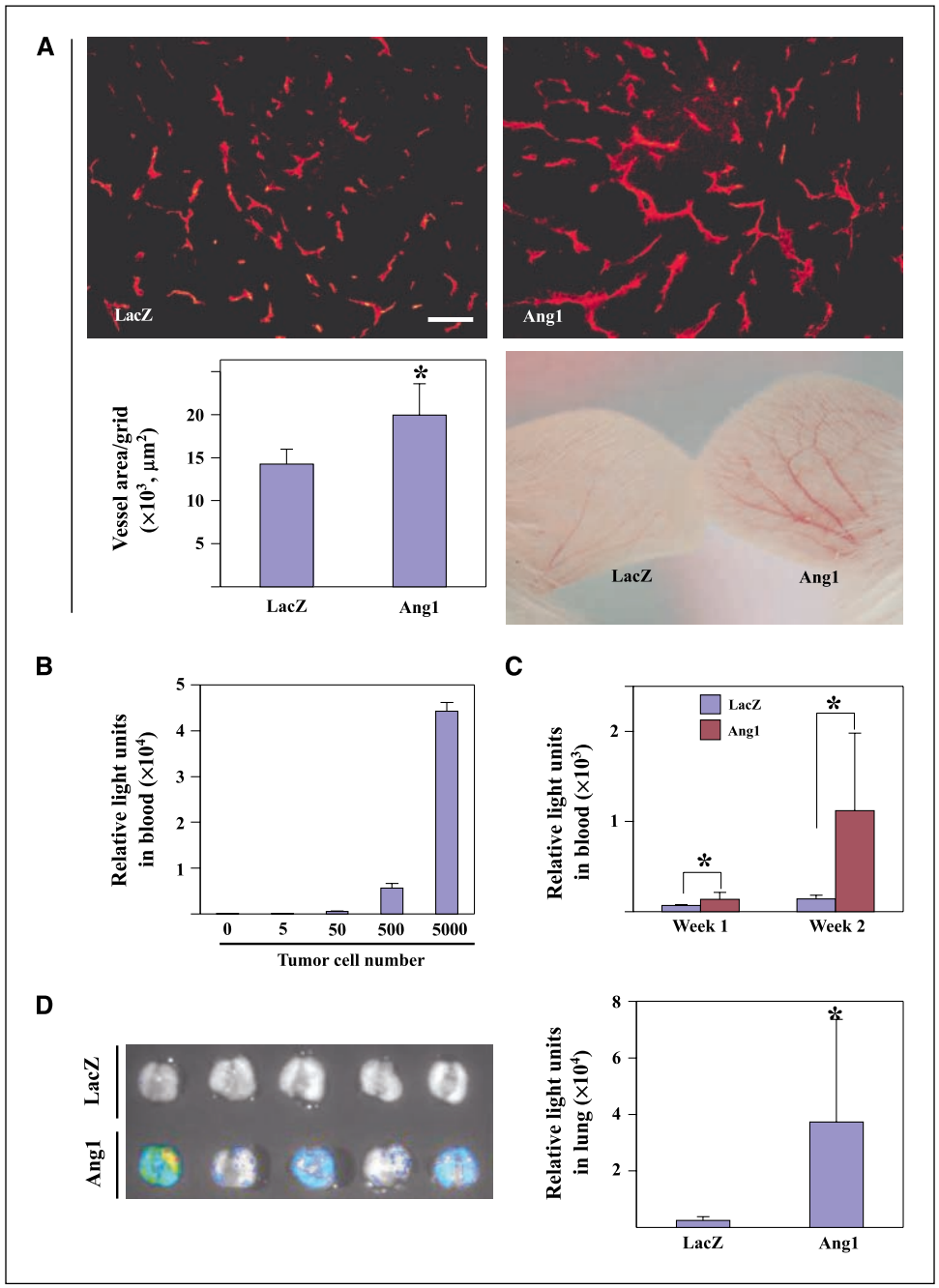

Figure 2. Ang1 promotes tumor dissemination by inducing vessel enlargement. *A,* immunohistochemical analysis of tumor vessels in LacZ- and Ang1-treated mice and quantification of the PECAM1-stained vessel area (Ang1 versus control: $P = 0.0131$). Vessel enlargement in the ear of an Ang1-treated mouse was shown in comparison with the control. *B,* titration of the minimal cell numbers detectable in blood by the luciferase assay system. When tumor cells from 5 to 5,000 cells were included for the measurement, the bioluminescence reading is 78.33 ± 6.66, 580.67 ± 90.14, 5,638.0 ± 1,031.90, and 44,283.67 ± 1,924.13, respectively ($n = 3$ for each point). *C,* quantification of bioluminescence in the blood. *D,* representative bioluminescent images of lungs with metastasis from mice treated with AdLacZ or AdAng1 and quantification of lung luminescence (AdAng1 versus AdLacZ: AdP = 0.0171). *, $P < 0.05$. *Bar,* 100 μm.

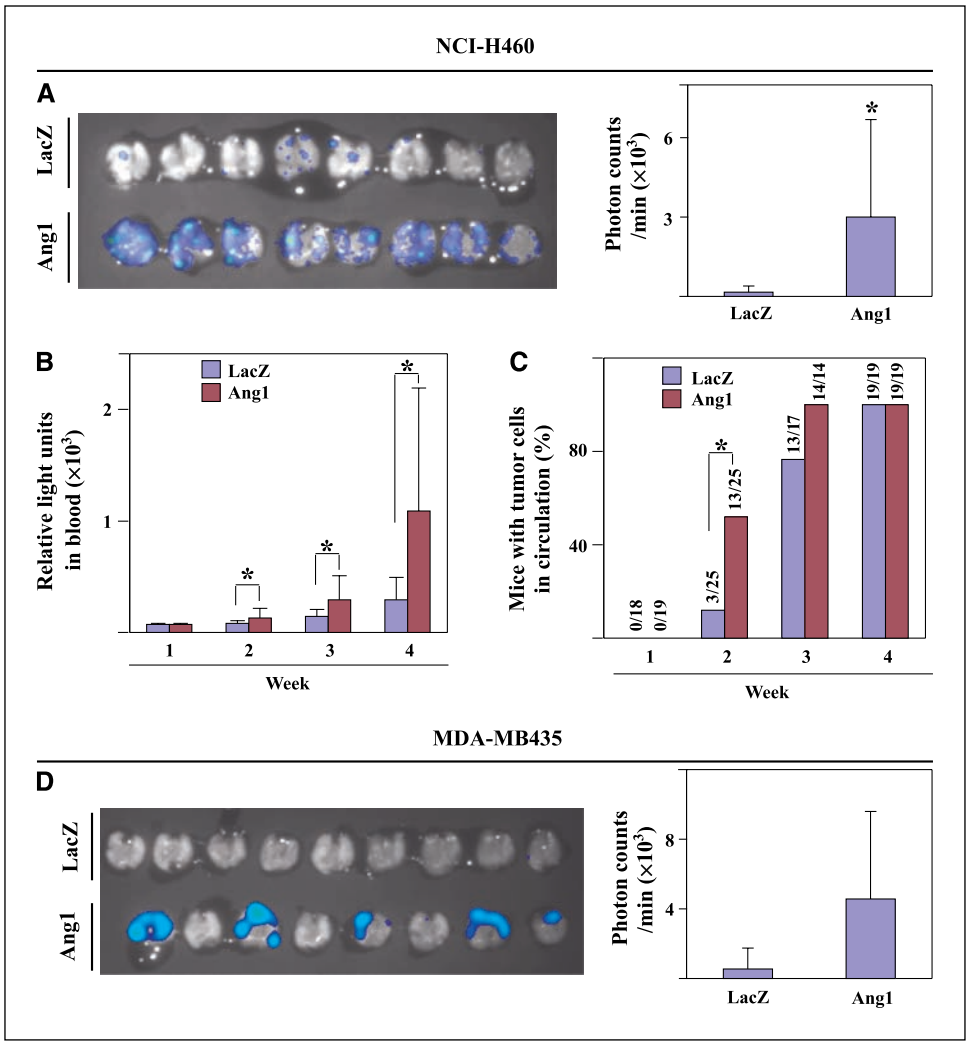

Figure 3. Ang1 accelerates the development of lung metastasis. *A,* representative bioluminescent images of lungs with metastasis from NCI-H460/Luc tumor–bearing mice treated with AdLacZ or AdAng1 and quantification of lung bioluminescence (AdAng1 versus AdLacZ: $P = 0.0083$). *B,* quantification of bioluminescence in blood (in relative light units). Week 1: AdAng1, 72.9 ± 7.72, $n = 19$; AdLacZ, 73.7 ± 8.71, $n = 18$. Week 2: AdAng1, 130.1 ± 83.53, $n = 25$; AdLacZ, 82.2 ± 24.84, $n = 25$, $P = 0.0084$. Week 3: AdAng1, 293.6 ± 214.28, $n = 14$; AdLacZ, 146.1 ± 57.90, $n = 17$, $P = 0.011$. Week 4: AdAng1, 1,090.0 ± 1,102.58, $n = 19$; AdLacZ, 291.8 ± 201.24, $n = 19$, $P = 0.0037$. *C,* percentage of mice with tumor cells in the circulation. *D,* representative bioluminescent images of lungs with metastasis from MDA-MB435/Luc tumor–bearing mice treated with AdLacZ or AdAng1 and quantification of lung bioluminescence. *, $P < 0.05$.

from the mice treated with AdAng1 and AdLacZ. Because most of the mice treated with AdAng1 died at about 4 weeks after surgical removal of primary tumors due to massive metastatic tumor burden in the lungs, mice were subsequently analyzed 3 weeks after tumor excision. Lungs with metastatic tumor nodules were weighed, and a statistically significant increase in lung weight was observed in the Ang1-treated versus control group (AdLacZ: 0.20 ± 0.05 g, $n = 17$; AdAng1: 0.67 ± 0.31 g, $n = 20$; $P < 0.0001$; Fig. 1*B*).

To confirm that the increase in tumor metastasis was due to Ang1, tumor-bearing mice receiving AdAng1 were simultaneously treated with AdsTie2. The circulating level of sTie2 1 week after the treatment was 0.42 ± 0.24 μg/mL ($n = 15$), which inhibited Ang1-induced Tie2 activation in the lungs (Supplementary Fig. S1). Tumors were s.c. implanted and analyzed as described above. Representative images of lungs from the mice treated with AdAng1 + AdsTie2 are shown in Fig. 1*A*. sTie2 suppressed the Ang1-induced increase of metastatic tumor burden in the lungs (AdAng1 + AdsTie2: 0.19 ± 0.01 g, $n = 7$; Ang1 versus Ang1 + sTie2: $P = 0.0004$; Fig. 1*B*). However, there was no significant difference in tumor weight between the Ang1-treated and control groups (AdLacZ: 1.80 ± 0.30 g, $n = 21$; AdAng1: 1.74 ± 0.32 g, $n = 24$), whereas the tumor weight was slightly but significantly decreased in mice treated with AdAng1 + AdsTie2 (1.55 ± 0.26 g, $n = 11$; AdAng1 + AdsTie2 versus AdLac: $P = 0.03$; Fig. 1*C*). Furthermore, histologic analysis confirmed that in tumor-bearing mice treated with AdAng1, the lungs were heavily occupied by the metastatic tumor cells in comparison with a few metastatic nodules in the control lungs (Fig. 1*D*).

Ang1 induces enlargement of tumor blood vessels to facilitate tumor cell dissemination. To understand the effect of Ang1 on tumor metastasis, the primary tumors from the treated and control mice were analyzed by immunohistochemical staining for PECAM-1 to visualize the tumor vasculature (Fig. 2*A*). PECAM-1–stained vessels in five microscopic fields of the highest vessel area (×200 magnification) were quantified using Image-Pro Plus. As shown in Fig. 2*A*, Ang1 increased vessel surface area significantly (AdLacZ: 14,246.20 ± 1,716.17 μm^2, $n = 5$; AdCA1: 19,962.89 ± 3,644.39 μm^2, $n = 5$; $P = 0.013$). However, there was no significant difference in PECAM-1^+ vessel counts per grid (Ang1: 117.68 ± 41.71, $n = 5$; LacZ: 108.84 ± 16.25, $n = 5$). As previously reported (31), Ang1-induced vessel enlargement was also observed in normal ear skin tissues (Fig. 2*A*).

To find out when the luciferase-expressing tumor cells start to invade into the circulation, blood from tumor-bearing mice was collected for the detection of luciferase activity. We first titrated the minimal cell number detectable by this assay. Blood was mixed with different numbers of luciferase expressing tumor cells for analysis (Fig. 2*B*). The baseline luminescence reading for blood samples without added tumor cells was 68.20 ± 12.40 RLU ($n = 5$).

Therefore, any reading above 100 RLU was considered as a positive signal. There was a barely detectable signal from 5 tumor cells in the assay, but luminescence signals could be readily detected when 50 cells were analyzed. In the Ang1-treated mice bearing LNM35/Luc tumors, tumor cells could be detected in the blood already at week 1 (4 of 8), whereas none of the nine mice in the control group gave positive signal (Fig. 2*C*, week 1; AdAng1: 138.0 ± 77.45 RLU, $n = 8$; AdLacZ: 68.78 ± 10.32 RLU, $n = 9$; $P = 0.018$). There was a dramatic increase of tumor cells in the blood from Ang1-treated mice at week 2 (Fig. 2*C*; AdAng1: 1,119.2 ± 860.51 RLU, $n = 5$; AdLacZ: 142.0 ± 41.45 RLU, $n = 8$; $P = 0.0071$). Consistent with this, tumor metastasis to lungs occurred in all Ang1-treated mice (5 of 5) when mice were sacrificed for analysis 2 weeks after tumor implantation, whereas only two of eight mice showed weak signals of metastasis in the lungs from the control group (Fig. 2*D*). Quantification of lung bioluminescence is shown in Fig. 2*D* (AdAng1: 37,363.60 ± 36,142.35 RLU, $n = 5$; AdLacZ: 2,466.0 ± 1,394.31 RLU, $n = 8$; $P = 0.017$).

Ang1 accelerates tumor metastasis development. To investigate whether Ang1 has similar effects in a poorly metastatic

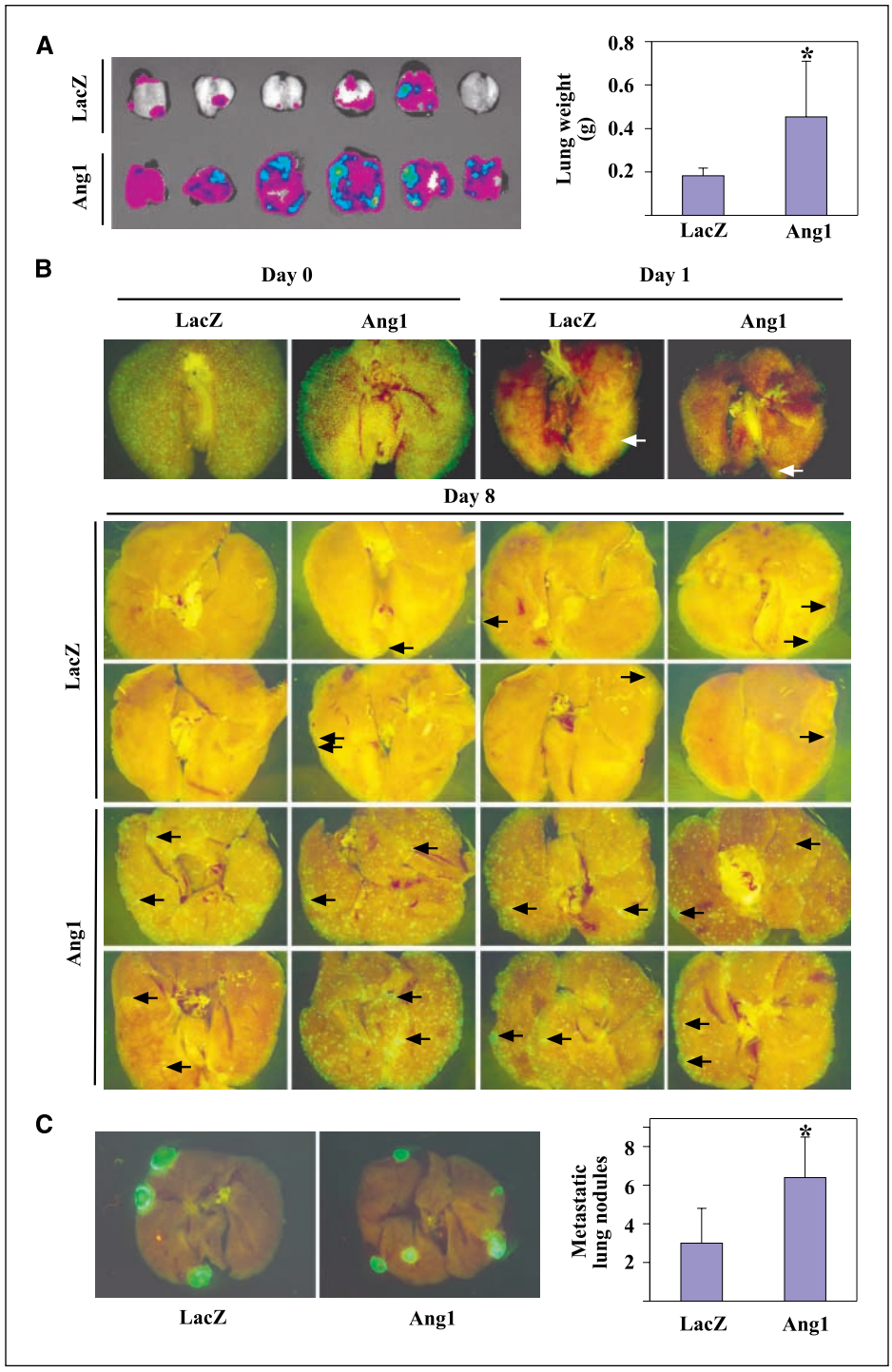

Figure 4. Ang1 promotes the establishment of metastatic foci in the lungs. *A,* representative images of the lungs with metastasis after i.v. injection of LNM35/Luc tumor cells and quantification of lung weight from the treated and control mice ($P = 0.0307$). *B,* analysis of metastatic tumor formation at different stages after i.v. injection of LNM35/GFP tumor cells (day 0, day 1, and day 8). *C,* analysis of Ang1 effect on tumor metastasis formation after surgical removal of primary tumors and quantification of metastatic nodules (GFP^+) in lungs (AdAng1 versus AdLacZ: $P = 0.0374$). *, $P < 0.05$.

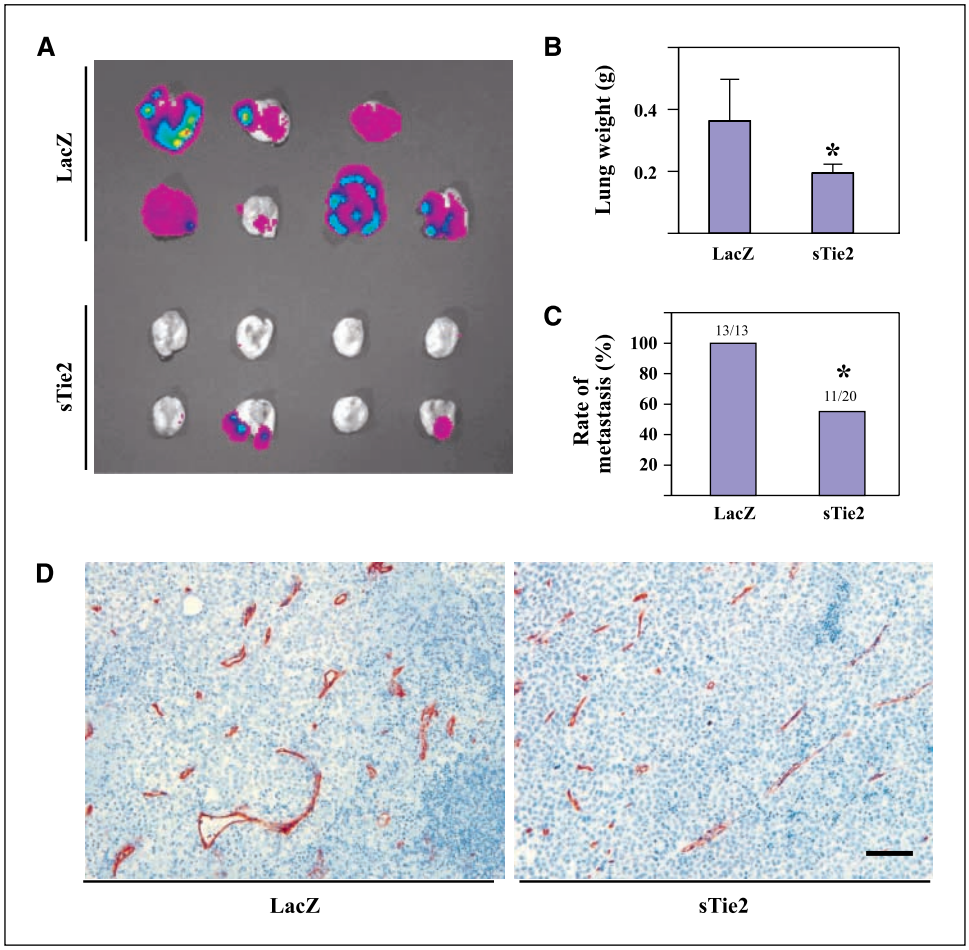

Figure 5. Suppression of lung metastasis by blocking Tie2 signaling. *A,* representative bioluminescent images of lungs from tumor-bearing mice treated with AdLacZ or AdsTie2. *B,* quantification of lung weight from the treated and control mice (AdsTie2 versus AdLacZ: $P = 0.0001$). *C,* rate of lung metastasis in mice treated with AdLacZ versus AdsTie2 ($P = 0.005$). *D,* immunohistochemical staining for PECAM-1 with tumor sections from LacZ- and sTie2-treated mice. *, $P < 0.05$. *Bar,* 50 μm.

tumor, NCI-H460/Luc tumor cells were implanted s.c. and the mice were treated with Ang1 as described above. Shown in Fig. 3*A* are representative bioluminescent images of lungs from the tumor-bearing mice treated with AdLacZ or AdAng1 when the mice were sacrificed 4 weeks after tumor implantation. Quantification of bioluminescence showed that Ang1 significantly increased tumor metastasis to the lungs (photon counts per minute; AdAng1: 2,998.09 ± 3,685.26, $n = 11$; AdLacZ: 162.01 ± 229.52, $n = 14$; $P = 0.008$; Fig. 3*A*). Consistent with this, tumor cell load in the blood was increased significantly in the Ang1-treated mice starting from week 2 (Fig. 3*B*). Furthermore, tumor cell dissemination to the circulation occurred earlier in the Ang1-treated mice. There was no detectable signal in the blood at week 1 in either the treated or control groups. However, more than half of the Ang1-treated mice (13 of 25) gave a positive signal in the blood, whereas only 3 of 25 mice gave a signal in the control group ($P = 0.0054$; Fig. 3*C*).

To further validate the above observation, we also used the slow-growing MDA-MB435/Luc melanoma model. Tumor cells were implanted s.c. and grown for 5 weeks before treatment with AdAng1. Ang1 increased tumor metastasis to the lungs when the mice were analyzed at week 9. Shown in Fig. 3*D* are representative bioluminescent images of the lungs. Quantification of lung metastasis as photon counts per minute (AdAng1: 4,568.85 ± 5,031.62, $n = 8$; AdLacZ: 539.06 ± 1,216.07, $n = 7$; $P = 0.0602$; Fig. 3*D*) indicated increased metastasis. However, this was not statistically significant due to a large individual variation, unless the highest and lowest values were excluded (AdAng1: 3,377.39 ± 2,150.84 $n = 6$; AdLacZ: 91.16 ± 67.76, $n = 5$; $P = 0.0081$). Quantification of blood bioluminescence revealed a significant increase of tumor cells in the blood from Ang1-treated mice at week 9 (AdAng1: 716.5 ± 634.35 RLU, $n = 16$; AdLacZ: 257.3 ± 282.34 RLU, $n = 15$; $P = 0.016$).

Ang1 enhances the establishment of tumor metastasis in the lungs. To investigate the effect of Ang1 on the establishment of tumor metastasis, mice were treated with AdAng1 or AdLacZ 2 days before i.v. injection of LNM35/Luc tumor cells and analyzed 4 weeks later. Shown in Fig. 4*A* are representative images of the lungs with metastasis. There was a significant difference in the lung weight between the treated and control groups (AdLacZ: 0.18 ± 0.04 g, $n = 6$; AdAng1: 0.45 ± 0.26 g, $n = 6$; $P = 0.031$; Fig. 4*A*).

In separate experiments, LNM35/Luc tumor–bearing mice were i.v. injected with LNM35/GFP tumor cells at week 3 after the tumor implantation and AdAng1 treatment. There was no obvious difference in initial number of tumor cells in the lungs between Ang1-treated and LacZ control groups when mice were analyzed immediately after tumor cell injection (Fig. 4*B*). However, more green fluorescent protein (GFP)–positive cells could be seen in lungs of Ang1-treated mice 1 day later (Fig. 4*B*), and this became more obvious when mice were analyzed at day 8 (Fig. 4*B*).

To further validate the role of Ang1 in the establishment of tumor metastasis, mice were treated with AdAng1 and AdLacZ after surgical removal of the primary LNM35/GFP tumors at week 3 and analyzed for metastasis in the lungs 3 weeks after the treatment. Shown in Fig. 4*C* are representative images from

AdLacZ- and AdAng1-treated mice. There was a significant increase of metastatic nodules in the Ang1-treated versus control mice ($P = 0.0374$).

Blockade of Tie2 signaling inhibits lung metastasis. We found that tumor cells used here expressed Ang1 but not Ang2 transcripts, and both Ang1 and Ang2 expression could be detected in tumor-associated mouse stromal cells (Supplementary Fig. S2). To investigate the role of tumor-derived angiopoietins in tumor progression, mice were treated with AdsTie2 or AdLacZ 1 day after implantation of the highly metastatic LNM35/Luc cells. The mice were analyzed within 4 weeks after tumor excision using the IVIS Imaging system. Bioluminescent signals emitted from the lungs of tumor-bearing mice (Fig. 5*A*) were quantified in photons per second ($\times 10^7$, mean ± SD; AdLacZ: 39.64 ± 52.06, $n = 7$; AdsTie2: 11.92 ± 19.44, $n = 17$). Lungs with metastatic nodules were collected and weighed (AdLacZ: 0.36 ± 0.13 g, $n = 7$; AdsTie2: 0.19 ± 0.03 g, $n = 17$; Fig. 5*B*). There was a significant difference in lung weight ($P = 0.0001$; Fig. 5*B*) and in the occurrence of lung metastasis between the AdsTie2 and AdLacZ groups [LacZ: 13 of 13 (100%); sTie2: 11 of 20 (55%); $P = 0.005$; Fig. 5*C*].

Tumors from mice treated with AdsTie2 or AdLacZ were surgically removed at week 3 and weighed. There was no significant difference in tumor weight between the treated and control mice (LacZ: 1.92 ± 0.28 g, $n = 12$; sTie2: 1.86 ± 0.34 g, $n = 21$). As shown by PECAM-1 immunostaining of tumor sections, there was no obvious difference in vessel density between the sTie2-treated and control tumors (Fig. 5*D*). However, there was a trend toward decreased vessel surface area in the sTie2-treated group (LacZ: 13,812.20 ± 4,429.91 μm^2, $n = 6$; sTie2: 11,177.08 ± 3,769.72 μm^2, $n = 6$).

Blockade of Tie2 signaling suppresses lymph node metastasis but not tumor lymphangiogenesis. To investigate the role of angiopoietins in tumor lymphangiogenesis and lymphatic metastasis, LNM35/Luc tumor–bearing mice were treated with AdsTie2 or AdLacZ. Primary tumors were surgically excised and tumor metastasis in the lymph nodes was analyzed at week 7, as described above. Shown in Fig. 6*A* are representative images of axillary lymph nodes from tumor-bearing mice treated with AdsTie2 or AdLacZ. Bioluminescent signals from lymph nodes were quantified (photons per second $\times 10^7$, mean ± SD; AdLacZ: 58.93 ± 83.13, $n = 7$; AdsTie2: 0, $n = 17$; AdLacZ versus AdsTie2: $P = 0.0063$; Fig. 6*A*). The occurrence of lymph node metastasis is shown in Fig. 6*B* [LacZ: 17 of 30 (56.7%); sTie2: 0 of 20 (0%)]. The results indicate that treatment with sTie2 significantly decreased the rate of lymph node metastasis compared with AdLacZ control ($P < 0.0001$).

Consistently, systemic treatment with Ang1 promoted lymph node metastasis. The rate of lymph node metastasis in mice treated with AdAng1 (17 of 19, 89.5%) was significantly increased in comparison with the AdLacZ group (17 of 30, 56.7%; AdAng1 versus AdLacZ: $P = 0.025$), and a simultaneous treatment with AdsTie2 suppressed AdAng1-induced increase of lymph node

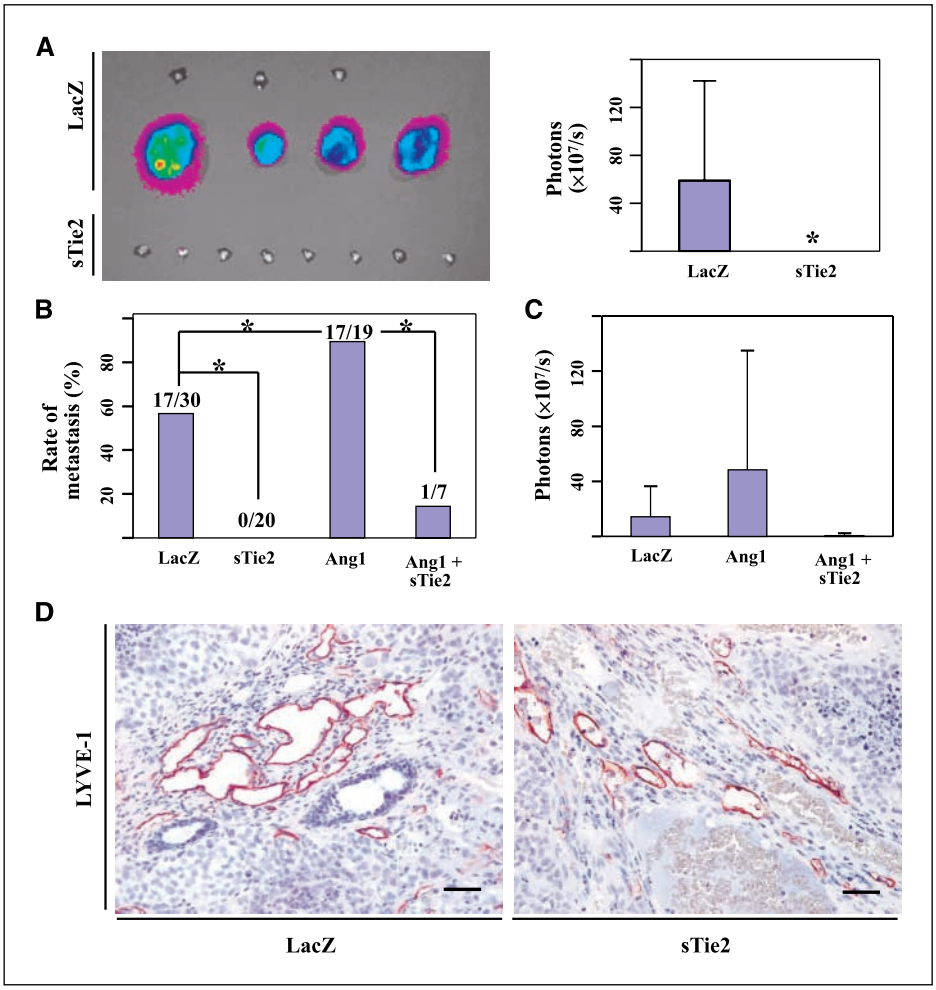

Figure 6. Inhibition of lymph node metastasis by blocking Tie2 signaling. *A,* representative images of axillary lymph nodes from tumor-bearing mice treated with AdLacZ or AdsTie2 and quantification of bioluminescent signals from the lymph nodes (AdsTie2 versus AdLacZ: $P = 0.0063$). *B,* rate of lymph node metastasis (sTie2 versus LacZ: $P < 0.0001$; Ang1 versus LacZ: $P = 0.0247$; Ang1 + sTie2 versus Ang1: $P = 0.0008$). *C,* quantification of bioluminescent signals from the lymph nodes. *D,* immunohistochemical analysis of tumor-associated lymphatic vessels in mice treated with AdsTie2 or AdLacZ. *, $P < 0.05$. *Bar,* 50 μm.

metastasis [1 of 7 (14.3%); AdAng1 + AdsTie2 versus AdAng1: $P = 0.0008$; Fig. 6*B*]. Bioluminescent signals emitted from the lymph nodes were quantified at week 6: AdLacZ (14.23 ± 22.06, $n = 17$), AdAng1 (48.43 ± 86.52, $n = 19$), or AdAng1 + AdsTie2 (0.62 ± 1.64, $n = 7$; photons per second $\times 10^7$; Fig. 6*C*). These values indicate a trend toward increased metastatic tumor burden in the lymph nodes of AdAng1 treated mice, and the effect could be suppressed by the simultaneous treatment with sTie2.

Lymphatic vessel density obtained by quantification of LYVE-1–positive vessels in three microscopic fields of the highest vessel density was 10.03 ± 1.70 in AdsTie2-treated tumors ($n = 8$) and 9.40 ± 1.70 in the control ($n = 7$). This indicated that tumor-associated lymphangiogenesis was not suppressed by AdsTie2 treatment (Fig. 6*D*).

Discussion

In this study, we show that systemic treatment with Ang1 increases tumor metastasis to the lungs through enhancement of tumor cell dissemination to the circulation and through promotion of the establishment of metastatic foci. Consistently, we confirmed that blockade of Tie2 signaling by the treatment with the soluble Tie2 receptor suppresses spontaneous and experimental tumor metastasis to the lungs and also the Ang1-induced increase in tumor metastasis. In spite of the strong effects of Tie2 signals on tumor metastasis, tumor growth and vessel density were not significantly affected. This suggests that the Ang1/Tie2 pathway regulates the properties of endothelial cells in the enlarged vessels to facilitate tumor progression.

It has been shown that transgenic overexpression of Ang1 in mouse skin results in enlargement of vessel size without increase in angiogenic sprouting (36, 37). Treatment with COMP-Ang1, a modified version of Ang1 with increased potency and solubility, also induced vascular enlargement and enhanced blood flow (31). Consistent with these findings, we observed here that Ang1 could induce vessel enlargement in both tumor and normal tissues. The vessel dilation effects of Ang1 may increase tumor cell dissemination and the establishment of metastatic foci in distant organs. Indeed, we found that systemic treatment with Ang1 promoted tumor metastasis to the lungs when tumors were s.c. implanted and also when the tumor cells were injected via the tail vein. The metastasis-promoting effect of Ang1 was not direct on tumor cells per se because Tie2 transcripts could not be detected by reverse transcription-PCR (RT-PCR) in the tumor cells, and *in vitro* treatment of the tumor cells with Ang1 did not promote their growth.[5] It has recently been shown that Ang1 could up-regulate hepatocyte growth factor (HGF) in endothelial cells (38). HGF, via signaling through MET, is known to promote normal and neoplastic invasive growth (39). However, we did not detect increased HGF expression in lungs by immunostaining or RT-PCR from Ang1-treated mice in comparison with the control.[6]

In our previous study using LNM35/Luc cells, it was rare to find tumor metastases in lung if primary tumors were surgically excised 2 weeks after tumor implantation (28). Here we observed that tumor metastasis occurred much earlier in the Ang1-treated mice, and tumor cell invasion into the circulation could be detected even 1 week after tumor implantation. Consistently, all mice treated with Ang1 had metastatic lesions in lungs when mice were analyzed 2 weeks after tumor implantation. This effect is specific because Ang1-induced increase in tumor metastasis could be suppressed by the simultaneous treatment with sTie2 (20). Surprisingly, the effect of Ang1 treatment on tumor metastasis could also be reproduced in mice implanted with poorly metastatic or slow-growing tumors. This suggests that the effect of Ang1 on tumor metastasis is independent of the invasion capacity of tumor cells.

Suppression of lung metastasis by the systemic Tie2 inhibition shown here is consistent with previous studies (20), but there was little effect on tumor growth. Although immunostaining with PECAM-1 did not reveal obvious difference in vessel density between the treated and control tumors, the vessel surface area seemed to be decreased by the treatment with sTie2. This suggests that Tie2 signaling is not required for the angiogenic sprouting of tumor-associated blood vessels, but rather has a role in regulating vessel diameter and properties that promote tumor metastasis.

Although Ang1 has been shown to be lymphangiogenic and Ang2 plays an important role in lymphatic development during embryogenesis (17–19), the role of Tie2 signaling in tumor lymphangiogenesis and lymphatic metastasis has not been studied before. We found that systemic treatment with sTie2 dramatically suppressed lymphatic metastasis. However, tumor-associated lymphangiogenesis was not affected by this treatment. This suggests a different mechanism from that of VEGF receptor-3 inhibition, which suppressed tumor-associated lymphangiogenesis (28, 34, 40–42). In agreement with the sTie2-mediated inhibition, treatment with Ang1 increased the rate of lymph node metastasis. This suggests that the Ang1/Tie2 signaling pathway is important for tumor cell dissemination via lymphatic vessels and the establishment of tumor metastasis in lymph nodes.

In summary, we have shown that systemic treatment with Ang1 promoted the formation of metastatic foci in lungs and lymph nodes, and that blockade of the Tie2 pathway could significantly suppress both hematogenous and lymphatic tumor metastasis. This suggests that targeting the Ang1/Tie2 signaling pathway could be an effective therapy for the treatment of metastatic diseases. Furthermore, because Ang1 has been shown to promote wound healing (43) and because it provides a potential factor for the treatment of cardiovascular diseases, our studies suggest that it may be better suited for local rather than systemic delivery to avoid the metastasis-promoting effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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[5] Our unpublished results.
[6] Unpublished data.

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