

## Angiopoietin-1 and -2 Exert Antagonistic Functions in Tumor Angiogenesis, yet Both Induce Lymphangiogenesis

Ernesta Fagiani, Pascal Lorentz, Lucie Kopfstein, and Gerhard Christofori

### Abstract

Members of the Angiopoietin family regulate various aspects of physiologic and pathologic angiogenesis. Although Angiopoietin-1 (Ang-1) decreases endothelial cell permeability and increases vascular stabilization via recruitment of pericytes and smooth muscle cells to growing blood vessels, Angiopoietin-2 (Ang-2) mediates angiogenic sprouting and vascular regression. In this study, we used the Rip1Tag2 transgenic mouse model of pancreatic  $\beta$ -cell carcinogenesis to investigate the roles of Ang-1 and Ang-2 in tumor angiogenesis and tumor progression. On their own, transgenic expression of human Ang-1 or Ang-2 in pancreatic  $\beta$  cells caused formation of peri-insular lymphatic vessels in the absence of effects on blood vessel density, islet morphology, or physiology. When crossed to Rip1Tag2 mice, both Ang-1- and Ang-2-expressing  $\beta$ -cell tumors showed increased peritumoral lymphangiogenesis in the absence of metastasis to local lymph nodes or distant organs. There was no alteration in tumor outgrowth, blood vessel density, or vessel maturation in Ang-1-expressing tumors. In contrast, Ang-2-expressing tumors exhibited diminished pericyte recruitment to blood vessels that were dilated, nonfunctional, and highly permeable. These tumors were hemorrhagic, highly infiltrated by leukocytes, and impaired in outgrowth. Together, our findings establish that Ang-2 antagonizes Ang-1 function, leading to excessive vessel sprouting with impaired pericyte recruitment and vessel stabilization. The poor perfusion of immature blood vessels results in retarded tumor growth, defining an important pathophysiologic pathway required for efficient tumorigenesis. *Cancer Res*; 71(17); 5717-27. ©2011 AACR.

### Introduction

The transition from prevascular hyperplasia to highly vascularized, outgrowing tumors is referred to as the "angiogenic switch" (1). The requirement of active angiogenesis for tumor outgrowth has been repeatedly shown, for example, by depletion of angiogenic growth factors or by the specific targeting of the activities of their receptors (2-4). Many factors are involved in inducing tumor angiogenesis and, among them, members of the VEGF family, fibroblast growth factors (FGF), and several others have been extensively studied (2-4). By binding to their cognate receptor Tie-2, angiopoietins (Ang) have also been shown to exert critical roles during physiologic angiogenesis and tumor angiogenesis. The family includes 4 ligands (Ang-1 to 4) and 2 corresponding tyrosine kinase receptors (Tie-1 and Tie-2; ref. 5, 6). Whereas *Ang-3* and *Ang-4*, diverging gene counterparts of mice and human, respectively, are still poorly understood, Ang-1 and Ang-2

have been intensely studied. Both specifically bind Tie-2 receptor with comparable affinities (7). However, whereas Ang-1 is an agonist of Tie-2 receptor signaling, in most circumstances Ang-2 exerts an antagonistic function. Ang-1 is predominantly expressed by mesenchymal cells and acts in a paracrine manner by binding Tie-2 receptors expressed on endothelial cells. Ang-1-mediated Tie-2 activation promotes vessel assembly and maturation by mediating survival signals for endothelial cells and by stimulating the recruitment of mural cells (8). Ang-1-mediated vessel stabilization can be seen as vessel normalization (9).

In contrast, Ang-2 is mainly produced by endothelial cells and stored in Weibel-Palade bodies from which it is released by activating cues and acts as an autocrine antagonist of Ang-1-mediated Tie-2 activation (6, 10). Ang-2 thereby primes and activates vascular endothelium to respond to other angiogenic factors, such as VEGF, and induces vascular destabilization but also vascular sprouting and branching. However, Ang-2 has also been reported to activate Tie-2 signaling, yet the molecular basis for its dual function is not fully understood (11, 12). Forced expression of Ang-2 in different tumor cell lines has also resulted in varying outcomes: It either enhanced the growth of hypervascular, often more invasive tumors (13-16), or it suppressed tumor growth by disrupting adequate tumor angiogenesis and vessel maturation (17). On the other hand, tumor growth is impaired in *Ang-2*-deficient mice (18).

The roles of Ang-1 and Ang-2 in tumor angiogenesis and tumor outgrowth have not been directly compared in

**Authors' Affiliation:** Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Basel, Switzerland

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**Corresponding Author:** Gerhard Christofori, Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Mattenstrasse 28, CH-4058 Basel, Switzerland. Phone: 41-61-267-3562; Fax: 41-61-267-3566; E-mail: gerhard.christofori@unibas.ch

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stochastically growing tumors. Here, we have used the Rip1-Tag2 transgenic mouse model of pancreatic  $\beta$ -cell carcinogenesis to force the expression of Ang-1 and Ang-2 during multistage tumor development. In (RT2) transgenic mice, SV40 large T antigen (Tag) is expressed under the control of the rat insulin promoter (Rip1) specifically in the insulin-producing  $\beta$  cells of pancreatic islets of Langerhans (19). Several tumor stages can be distinguished, including  $\beta$ -cell hyperplasia, angiogenic dysplasia, adenoma, and carcinoma (20, 21). One of the factors inducing angiogenesis in tumors of Rip1Tag2 mice is VEGF-A. It is secreted by  $\beta$ -tumor cells, and matrix-associated VEGF-A is released by matrix metalloproteinase-9 (MMP-9) concomitantly with the onset of angiogenesis (22). Consistent with these findings, inhibition of VEGF-A or MMP function represses tumor angiogenesis (23–25).

To assess the functional contribution of Ang-1 and Ang-2 to tumor angiogenesis, we have generated transgenic mouse lines, where either human Ang-1 (hAng-1) or human Ang-2 (hAng-2) are expressed under the control of the Rip1 promoter [Rip1Ang-1 or Rip1Ang-2 (RA-1; RA-2)]. RA-1 or RA-2 mice were then crossed with RT2 mice. Transgenic expression of both Ang-1 and Ang-2 led to the formation of lymphatic vessels around islets of Langerhans and  $\beta$ -cell tumors. Although Ang-1 only marginally increased blood vessel maturation and tumor growth, the expression of Ang-2 led to increased numbers of infiltrating leukocytes, elevated levels of VEGF-A expression, aberrant tumor angiogenesis, and, with it, retarded tumor growth.

## Materials and Methods

### Mice

The generation of RA-1 and RA-2 single-transgenic mice and of RT2/RA-1 and RT2 RA-2 double-transgenic mice is described in Supplementary Material. RT2;RA-1 and RT2;RA-2 and RT2 littermate control mice were sacrificed between 12 and 13 weeks of age. PTK787/ZK222584 (PTK/ZK) was kindly provided by Novartis Pharma. Twelve-week-old RT2 or RT2; Rip1Ang-2 mice were treated with either 100 mg/kg body-weight PTK/ZK dissolved in polyethylene glycol 300 (PEG-300; Sigma-Aldrich) or PEG-300 alone by daily oral administration for 5 days. All experimental procedures involving mice were done according to the guidelines of the Swiss Federal Veterinary Office and the regulations of the Cantonal Veterinary Office of Basel Stadt.

### Histopathologic analysis

Processing of tissues for histologic analysis is described in Supplementary Material. Histologic analysis and immunostaining was done on paraffin sections (5  $\mu$ m) or on cryosections (7  $\mu$ m) as previously described (26, 27). For 3-dimensional confocal reconstruction images, pancreatic cryosections (80–100  $\mu$ m) were used (28). Antibodies used are described in Supplementary Material. CD31<sup>+</sup> intratumoral endothelial cells and CD45<sup>+</sup>, F4/80<sup>+</sup>, 7/4<sup>+</sup>, pH3<sup>+</sup>, or caspase-3<sup>+</sup> cells in the tumors were determined using Image J software (National Institute of Mental Health). Histologic staging and grading of tumors were done as described (20).

### Lectin perfusion

For the analysis of functional blood vessels, mice were tail vein-injected with 100  $\mu$ L of 1 mg/mL fluorescein-labeled *Lycopersicon esculentum* lectin (FL-1171, Vector Laboratories) under intraperitoneal anesthesia with Nembutal Sodium Solution (Abbott Laboratories). After 5 minutes, mice were heart perfused with 10 mL of 4% PFA followed by 10 mL of PBS. Isolated pancreata were immersed in ascending concentrations of sucrose (12%, 15%, and 18% for 1 hour each at +4°C) overnight and embedded in ornithine carbamyl transferase.

### Cell lines

Human umbilical vein endothelial cells (HUVEC) were purchased from American Type Culture Collection and used at low passage numbers and without further authentication for the collagen gel assay described in Supplementary Methods.

### Statistical analyses

Statistical analysis was done using GraphPad Prism software (GraphPad Software Inc.).

## Results

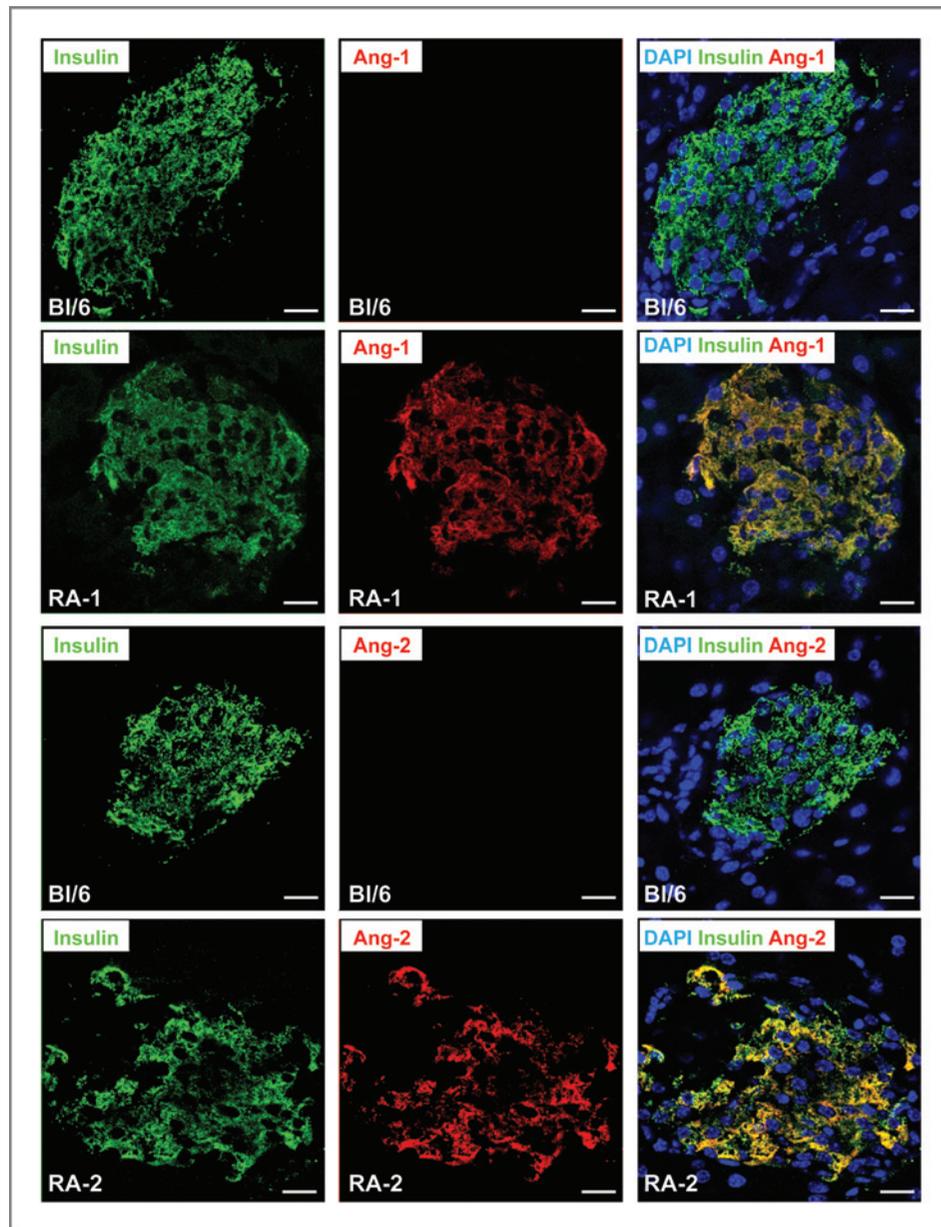
### Transgenic expression of human Ang-1 and Ang-2

Quantitative reverse transcription PCR (qRT-PCR) and immunoblotting analysis revealed that endogenous mouse Ang-1 and Ang-2 were expressed at low levels in the  $\beta$  cells of pancreatic islets of Langerhans or in tumors of RT2 mice (Supplementary Figs. S1 and S2A). To assess whether angiopoietins could have a role in tumor angiogenesis in RT2 mice we targeted expression of hAng-1 and hAng-2 to the  $\beta$  cells of pancreatic islets of Langerhans. cDNA fragments encoding hAng-1 and hAng-2, fused in frame at their 5' ends with a signal peptide and a 10-amino acid Myc-tag were cloned between the rat insulin II gene promoter fragment Rip1 (19) and a SV40 polyadenylation site (Ref. 27; Supplementary Fig. S2B). Seven RA-1 and 6 RA-2 founder lines were analyzed for transgene expression by RT-PCR, immunoblotting, and immunofluorescence microscopy with specific antibodies against human angiopoietins and against Myc-tag. Three founder lines each exhibited specific expression of correct-size hAng-1 and hAng-2, respectively, in pancreatic  $\beta$ -cells (Fig. 1, Supplementary Figs. S1, S2A, and S3). No expression of the transgenes was detected in islets of wild-type littermate mice. Two founder lines each of RA-1 and RA-2 exhibited homogeneous expression of hAng-1 and hAng-2, respectively, in all  $\beta$ -cells of individual islets of Langerhans and were used for further experimentation.

### Peri-insular lymphangiogenesis in RA-1 and RA-2 transgenic mice

Histopathologic analysis of pancreata of RA-1 and RA-2 mice did not reveal any changes in the number or morphology of islets as compared with nontransgenic littermate mice. The ratio and localization of endocrine  $\alpha$  and  $\beta$ -cells within individual islets of Langerhans and fasting blood glucose levels were unaffected in RA-1 and RA-2 mice (data not shown).

**Figure 1.** Transgenic expression of hAng-1 and hAng-2. Immunofluorescence stainings of islets with anti-insulin antibodies (green) and with anti-human and mouse (h/m) Ang-1 antibodies (red) on pancreatic sections from C57Bl/6 and RA-1 transgenic mice (top) and of insulin (green) and h/mAng-2 (red) on pancreatic sections from C57Bl/6 and RA-2 transgenic mice (bottom). Nuclei are visualized by DAPI staining (blue). Bars, 15  $\mu$ m.



Immunofluorescence staining with antibodies against the endothelial cell surface marker CD31 revealed that neither the expression of Ang-1 nor of Ang-2 did significantly change blood vessel morphology or density in islets of transgenic mice as compared with nontransgenic controls (Supplementary Fig. S4A and B).

However, staining of pancreatic sections with antibodies against the lymphatic endothelial cell marker LYVE-1 revealed a significant increase in the density of lymphatic vessels in the periphery of islets of RA-1 and RA-2 mice as compared with nontransgenic controls (Fig. 2A and B). These results indicate that both Ang-1 and Ang-2 are able to induce lymphangiogenesis, an observation consistent with previous reports that mice deficient in Ang-2 expression exhibit defects in lymphangiogenesis and that Ang-1 is capable of inducing lymphatic sprouting and hyperplasia (29–31).

genesis and that Ang-1 is capable of inducing lymphatic sprouting and hyperplasia (29–31).

#### Ang-1 and Ang-2 affect tumor growth

To assess the role of Ang-1 and Ang-2 in tumorigenesis, RA-1 and RA-2 transgenic mice were crossed with RT2 mice. The resulting RT2 single-transgenic mice and RT2;RA-1 (RTRA-1) or RT2;RA-2 (RTRA-2) double-transgenic mice were sacrificed between 12 and 13 weeks of age, when these mice succumb to hypoglycemia caused by excessive tumoral insulin production. Immunofluorescence and immunoblotting analysis revealed that in RTRA-1 and RTRA-2 double-transgenic mice most  $\beta$ -tumor cells expressed hAng-1 and hAng-2, respectively (Supplementary Figs. S2B, S5, and S6), yet human or mouse

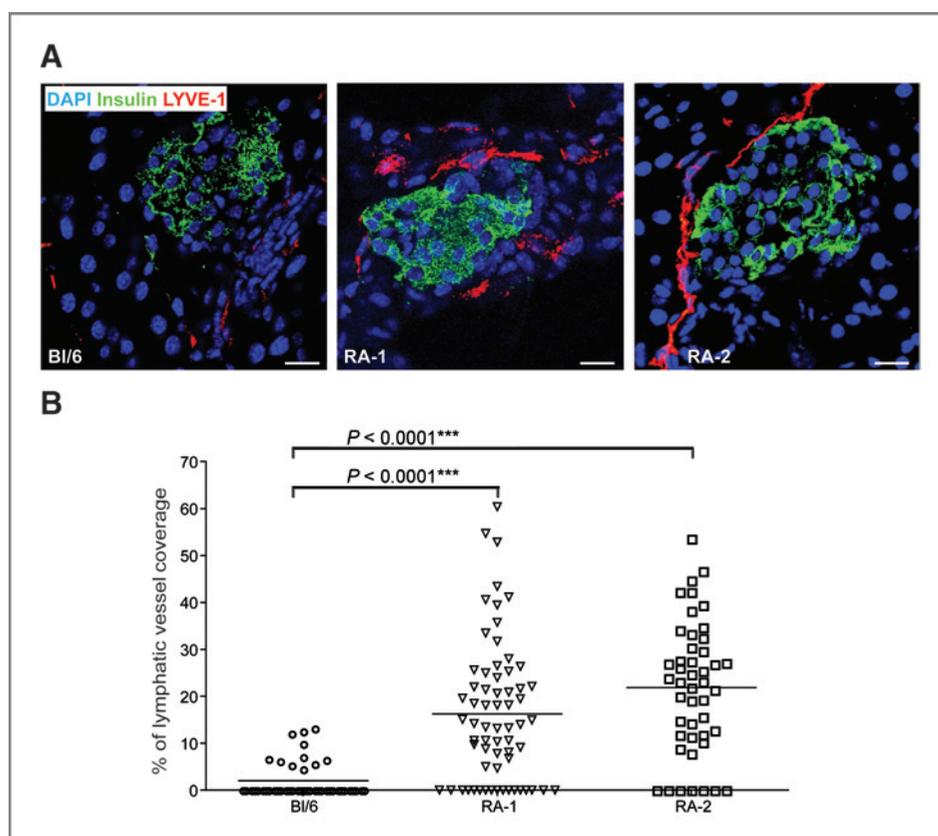


Figure 2. Increased peri-islet lymphangiogenesis in RA-1 and RA-2 transgenic mice. A, immunofluorescence staining of pancreatic islets from C57Bl/6, RA-1, and RA-2 mice with anti-insulin antibodies (green) and with anti-LYVE-1 antibodies (red). B, quantification of LYVE-1-positive peri-islet lymphatic vessels. The percentage of circumferential coverage by lymphatic vessels was determined for individual pancreatic islets in RA-1 ( $N = 9$ ,  $n = 65$ ) and in RA-2 ( $N = 10$ ,  $n = 46$ ) transgenic mice as compared with C57Bl/6 control mice ( $N = 9$ ,  $n = 44$ ). \*\*\*,  $P < 0.0001$ , Mann-Whitney test. Bars, 15  $\mu\text{m}$ .

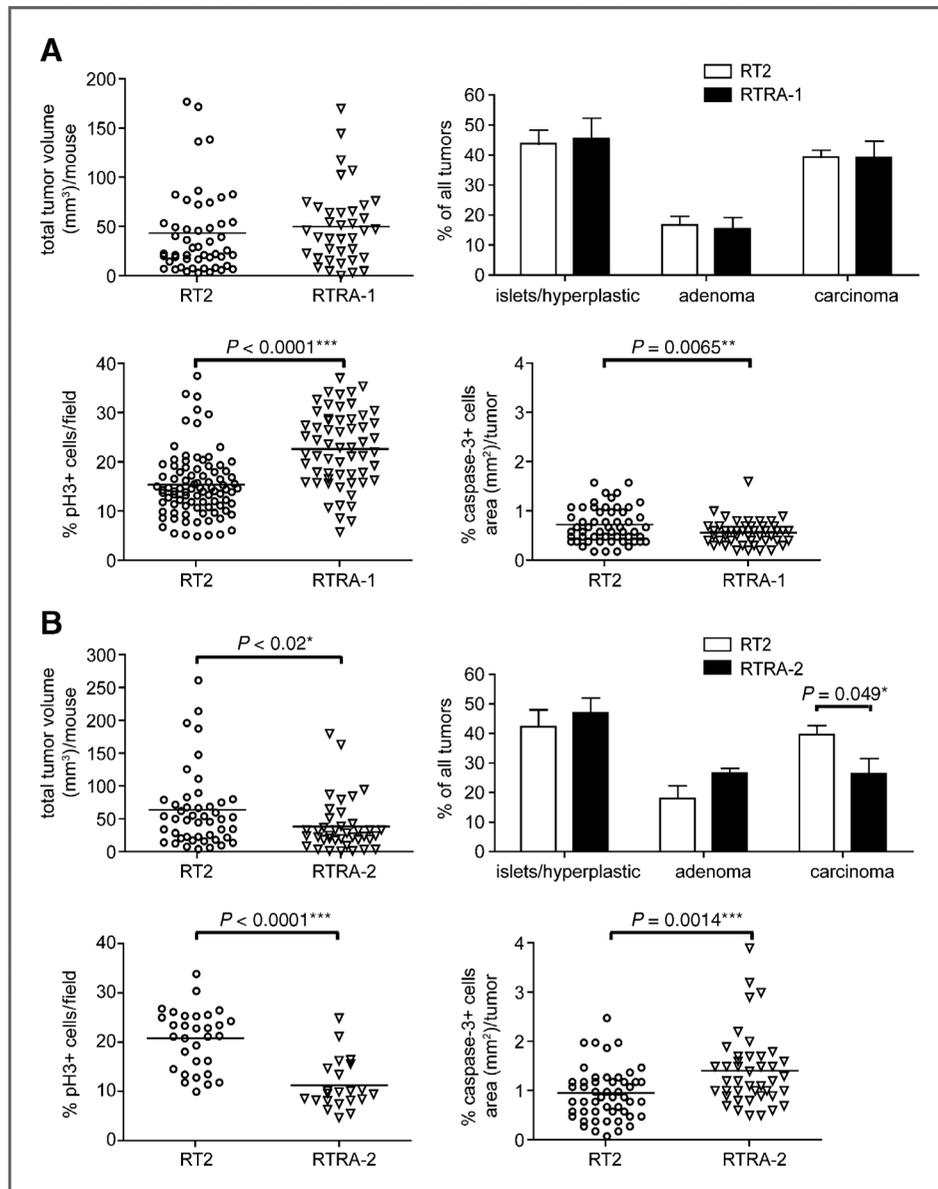
angiopoietins were not detectable by ELISA in the serum of these mice (data not shown).

Tumor growth in RTRA-1 mice was moderately, yet not significantly increased as compared with single-transgenic RT2 littermates (Fig. 3A). Histologic analysis of tumor progression revealed that the numbers of normal and hyperplastic islets, adenomas and carcinomas were unchanged (Fig. 3A). In contrast, RTRA-2 mice displayed a significant reduction in tumor volumes (Fig. 3B) and in the number of tumors per mouse ( $3.64 \pm 0.33$  for RTRA-2 vs.  $5.48 \pm 0.35$  for RT2). Furthermore, the fraction of hyperplastic islets and adenomas increased, whereas carcinomas were decreased in RTRA-2 mice (Fig. 3B). Stainings with antibodies against insulin on histologic sections of pancreas, liver, and lung did not reveal any metastasis in lymph nodes and lung (Supplementary Fig. S7A), whereas micrometastasis detectable in the livers of these mice was not affected by the transgenic expression of Ang-1 and Ang-2, respectively (Supplementary Fig. S7B).

Concomitant with the increased and reduced tumor growth in RTRA-1 and in RTRA-2 mice, respectively, the numbers of proliferating, phosphohistone-3-positive  $\beta$ -tumor cells were significantly increased in RTRA-1 and significantly decreased in RTRA-2 mice (Fig. 3A and B). In addition, analysis of apoptosis by staining for cleaved caspase-3 revealed a significant reduction in the number of apoptotic cells within the tumors of RTRA-1 and a significant increase in RTRA-2 mice (Fig. 3A and B).

#### Aberrant tumor angiogenesis in RTRA-2 mice

The slight increase and the significant decrease of tumor outgrowth in RTRA-1 and in RTRA-2 mice, respectively, may be based on alterations in tumor-induced angiogenesis. Immunofluorescence staining for CD31 revealed no differences in the number of intratumoral blood vessels in RTRA-1 as compared with tumors from RT2 mice (Fig. 4A). To further examine the functionality of blood vessels, the various genotype mice were injected i.v. with fluorescein-labeled *L. esculentum* lectin to visualize vessel perfusion and morphology. The tumor blood vessels in RTRA-1 and in RT2 control mice were fully perfused (Fig. 4A, Fig. 5A). In contrast, in RTRA-2 mice intratumoral blood vessels and endothelial cells were significantly increased in numbers, yet seemed dilated and disorganized, and their perfusion was dramatically impaired (Fig. 4B, Fig. 5A). Pericyte coverage as one hallmark of capillary maturation was analyzed by immunofluorescence staining with antibodies against NG2 and CD31 for pericytes and endothelial cells, respectively (Fig. 5B). In RTRA-1, pericyte density and the percentage of tumor blood vessels covered with pericytes did not differ from RT2 controls (Fig. 4A, Fig. 5B). However in RTRA-2 mice, overall pericyte density was slightly diminished and pericyte-blood vessel coverage was significantly decreased as compared with RT2 control mice (Fig. 4B, Fig. 5B). Three-dimensional reconstruction of laser-scanning confocal microscopic images confirmed the intimate connection of pericytes with endothelial cells in RT2 and RTRA-1 mice and the disruption of these interactions in RTRA-2 mice (Fig. 5B).

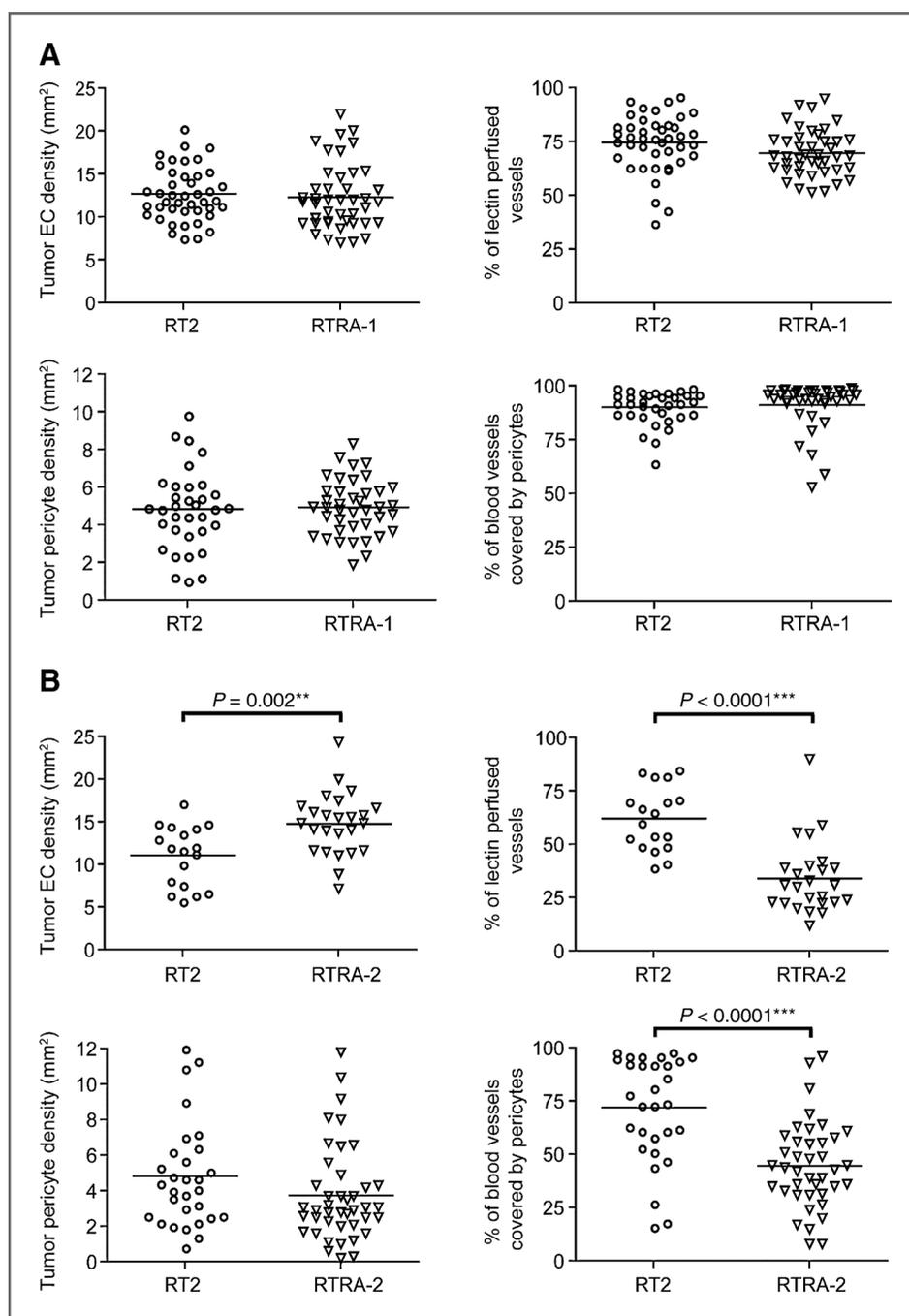


**Figure 3.** Tumor growth in RT2, RTRA-1, and RTRA-2 mice. **A**, top left, tumor volumes in RTRA-1 ( $N = 50$ ) as compared with RT2 ( $N = 37$ ) mice. Single points ( $n$ ) represent the total tumor volume per mouse. Top right, the percentages of normal/hyperplastic islets, adenomas, and carcinomas in RTRA-1 ( $N = 9$ ) and RT2 ( $N = 9$ ) mice. Bottom left, the proliferation rate of  $\beta$ -tumor cells as determined by immunofluorescence staining of pancreatic sections with anti-phospho-histone-3 antibodies in RTRA-1 ( $N = 3$ ,  $n = 90$ ) and RT2 ( $N = 2$ ,  $n = 59$ ) mice.  $^{***}$ ,  $P < 0.0001$  unpaired Student  $t$  test. Bottom right, the rate of  $\beta$ -tumor cell apoptosis as determined by immunofluorescence staining of pancreatic sections with antibodies against cleaved caspase-3 in RTRA-1 ( $N = 3$ ,  $n = 56$ ) and RT2 ( $N = 2$ ,  $n = 46$ ) mice.  $^{**}$ ,  $P = 0.0065$  unpaired Student  $t$  test. **B**, top left, tumor volumes in RTRA-2 ( $N = 39$ ) as compared with RT2 ( $N = 46$ ) mice.  $^{**}$ ,  $P = 0.018$  unpaired Student  $t$  test. Top right, the percentages of normal/hyperplastic islets, adenomas, and carcinomas in RTRA-2 and ( $N = 9$ ) and RT2 ( $N = 9$ ) mice.  $^*$ ,  $P = 0.049$  unpaired Student  $t$  test. Bottom left, the proliferation rate of  $\beta$ -tumor cells as determined by antiphosphohistone-3 staining in RTRA-2 ( $N = 3$ ,  $n = 22$ ) and RT2 ( $N = 4$ ,  $n = 31$ ).  $^{***}$ ,  $P < 0.0001$  unpaired Student  $t$  test. Bottom right, the rate of  $\beta$ -tumor cell as determined by anticlaved caspase-3 staining in RTRA-2 ( $N = 3$ ,  $n = 42$ ) and RT2 ( $N = 4$ ,  $n = 51$ ) mice.  $^{**}$ ,  $P = 0.0014$  unpaired Student  $t$  test.  $N =$  total number of mice,  $n =$  total number of microscopic fields (magnification  $\times 20$ ). Note that the average tumor volumes from the RT2 control mice are different in A and B, because littermate controls of each transgenic line have been analyzed to consider potential genetic background variations and genetic drifts.

To investigate whether Ang-1 or Ang-2 exerted a direct proangiogenic function, we cocultured isolated dysplastic islets in a collagen matrix together with HUVECs. In this assay, angiogenic dysplastic islets derived from RT2 mice induce HUVEC migration, proliferation, and tube formation, whereas nonangiogenic

islets fail to do so (21, 27). In this assay, islets from 9-week-old RT2, RTRA-1, and RTRA-2 mice did not show any significant difference in their angiogenic activities (Supplementary Fig. S8A–C).

Next, we assessed which cell types of the various genotype tumors expressed the receptor for angiopoietins. RT-PCR

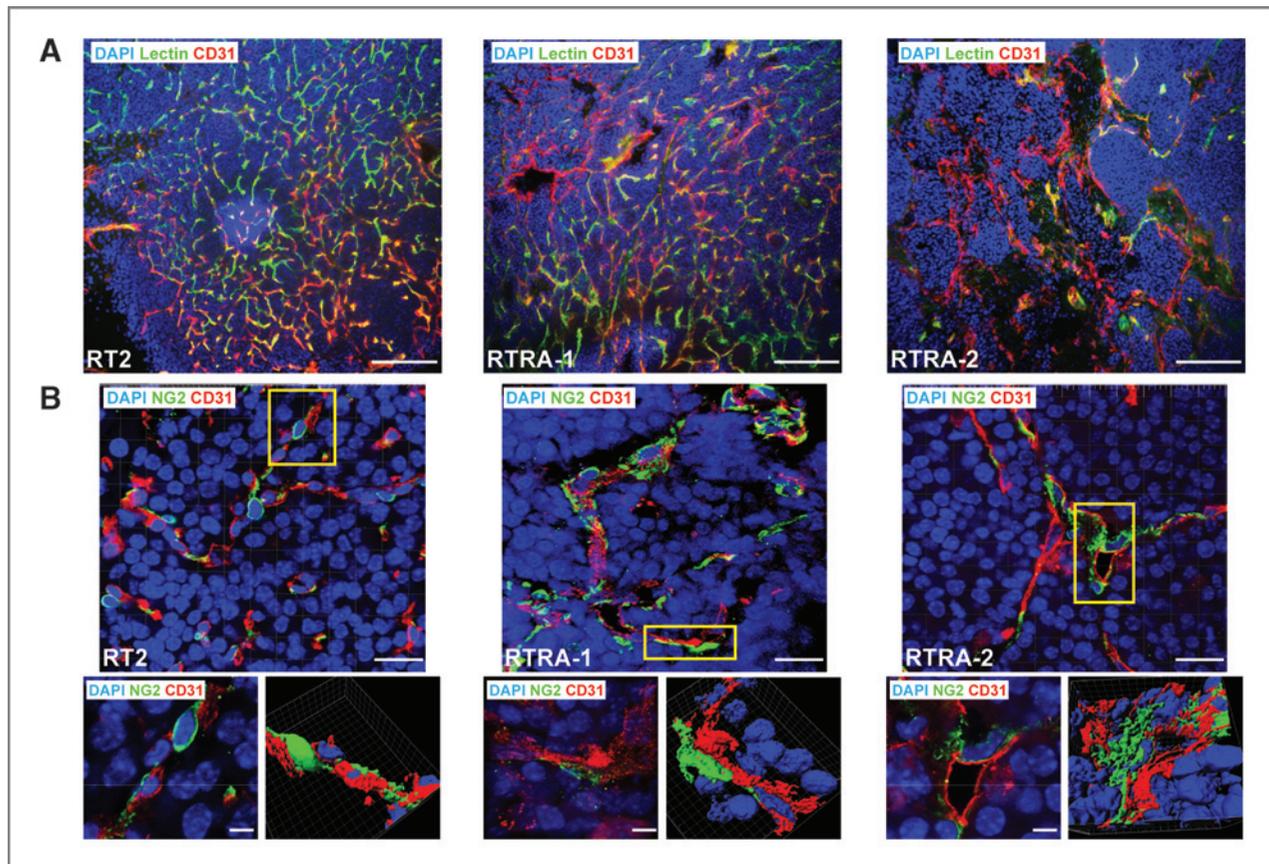


**Figure 4.** Significant changes in tumor angiogenesis by Ang-2 but not Ang-1. **A**, top left, tumor endothelial cell density determined as the percentage of CD31-positive area out of the total tumor area analyzed in RTRA-1 ( $N = 3$ ,  $n = 43$ ) and RT2 ( $N = 3$ ,  $n = 43$ ) mice. Top right, vessel perfusion quantified as the percentage of FITC-lectin-perfused vessels of the total number of CD31-positive endothelial cells per tumor area analyzed in RTRA-1 ( $N = 3$ ,  $n = 43$ ) and RT2 ( $N = 3$ ,  $n = 43$ ) mice. Bottom left, tumoral pericyte density evaluated as the percentage of NG2-positive cells out of the total tumor area analyzed in RTRA-1 mice ( $N = 8$ ,  $n = 40$ ) and RT2 mice ( $N = 6$ ,  $n = 34$ ). Bottom right, the percentage of CD31-positive endothelial cells associated with NG2-positive pericytes in RTRA-1 ( $N = 8$ ,  $n = 40$ ) as compared with RT2 ( $N = 6$ ,  $n = 34$ ) mice. **B**, top left, endothelial cell density in tumors of RTRA-2 ( $N = 3$ ,  $n = 25$ ) as compared with RT2 ( $N = 3$ ,  $n = 18$ ) mice. **\*\***,  $P = 0.002$  unpaired Student *t* test. Top right, functional blood vessels were quantified as in (A) in RTRA-2 ( $N = 3$ ,  $n = 25$ ) and RT2 ( $N = 3$ ,  $n = 18$ ) mice. **\*\*\***,  $P < 0.0001$  unpaired Student's *t* test. Bottom left, tumoral pericyte density in RTRA-2 ( $N = 4$ ,  $n = 42$ ) compared with RT2 ( $N = 4$ ,  $n = 30$ ) mice. Bottom right, pericyte-coverage of tumoral blood vessels in RTRA-2 ( $N = 4$ ,  $n = 42$ ) and RT2 ( $N = 4$ ,  $n = 30$ ) mice. **\*\*\***,  $P < 0.0001$  unpaired Student *t* test.  $N$  = number of mice,  $n$  = number of microscopic fields (magnification 20 $\times$ ).

analysis revealed that Tie-2 is expressed mainly by CD31<sup>+</sup> endothelial cells (BEC) and not by glucagon-like peptide receptor 1-positive (GLP-1R<sup>+</sup>)  $\beta$ -tumor cells sorted by FACS from RT2 tumors (Supplementary Fig. S9A). To examine whether Ang-1 or Ang-2 were able to activate Tie-2, proteins phosphorylated on tyrosine residues were immunoprecipitated from tumors of RT2, RTRA-1, and RTRA-2 mice, and phosphorylated Tie-2 was then detected by immunoblotting with antibodies against Tie-2 (Supplementary Fig. S9B). Phosphorylated Tie-2 was found at comparable levels among

the 3 genotypes of mice. This result was further confirmed by ELISA determining the levels of phospho-Tie-2 (Supplementary Fig. S9C) and by immunofluorescence analysis with an antibody specifically recognizing phospho-Tie-2 (Supplementary Fig. S10). Together, these analyses revealed that the transgenic expression of Ang-1 and Ang-2 did not significantly change the overall levels of Tie-2 receptor activation.

Previously, it has been reported that Ang-2 and VEGF-A act together to induce angiogenesis. In fact, Ang-2 by itself



**Figure 5.** Ang-1 promotes and Ang-2 prevents vessel maturation. **A**, immunofluorescence stainings of intratumoral endothelial cells with anti-CD31 antibodies (red) on pancreatic sections from FITC-lectin-perfused (green) RT2, RTRA-1, and RTRA-2 mice. Bars, 100 μm. **B**, immunofluorescence staining with antibodies against CD31 (red) and NG2 (green) on 80-μm thick pancreatic sections from RT2, RTRA-1, and RTRA-2 mice. Images were taken with a Leica SP5 laser-scanning confocal microscope. Bars, 20 μm. Boxes indicate regions of higher magnification of single blood vessels (bottom left; bars, 4 μm) and their 3-dimensional reconstructions using Imaris software (bottom right).

induces vessel regression in the absence of VEGF-A or upon inhibition of VEGF-A (32). To address whether Ang-2 cooperates with VEGF-A in modulating tumor angiogenesis in RTRA-2 mice, we determined VEGF-A mRNA and protein levels in tumors and serum of the various genotypes of mice (Supplementary Fig. S11A and B). Indeed, VEGF-A levels were significantly higher in RTRA-2 mice and lower in RTRA-1 mice as compared with RT2 control mice. To test the possibility that in RTRA-2 double-transgenic mice, VEGF-A and Ang-2 work synergistically to promote tumor angiogenesis, the small-molecule VEGF receptor inhibitor PTK787/ZK222584 (PTK/ZK; 33) was used to interfere with VEGF receptor function in RTRA-2 mice. PTK/ZK has been previously reported to efficiently repress tumor angiogenesis and tumor growth in the RT2 mouse model (24). Tumor growth and tumor angiogenesis were significantly repressed after treatment of 12-week-old RT2 and RTRA-2 mice with PTK/ZK for 5 days as compared with control-treated mice (Supplementary Fig. S12A–C). Notably, the increased endothelial cell numbers in tumors of RTRA-2 mice were substantially reduced upon PTK/ZK treatment compared with the levels found in RT2 tumors treated with PTK/ZK.

This result indicates that the Ang-2-mediated increase in endothelial cell density, as observed in RTRA-2 mice, relies on VEGF-A function. However, the cooperation between Ang-2 and VEGF-A leads to the generation of many non-functional vessels.

#### Inflammatory cell infiltration in tumors of RTRA-1 and RTRA-2 mice

VEGF-A is known to recruit bone marrow-derived cells to sites of active angiogenesis (34, 35). In particular, monocytes and macrophages are well known to contribute to tumor progression and tumor-associated angiogenesis (2, 36, 37). Immunofluorescence staining revealed that the numbers of tumor-infiltrating F4/80<sup>+</sup> macrophages and of 7/4<sup>+</sup> neutrophils were significantly reduced in RTRA-1 tumors and significantly increased in RTRA-2 tumors (Fig. 6A–D), whereas the overall numbers of CD45<sup>+</sup> inflammatory cells did not differ between tumors of RT2, RTRA-1, and RTRA-2 mice (Fig. 6C and D). However, neither Ang-2 nor Ang-1 were able to affect the infiltration of macrophages or neutrophils into islets of RA-1 and RA-2 mice as compared with C57Bl/6 mice (Supplementary Fig. S13).

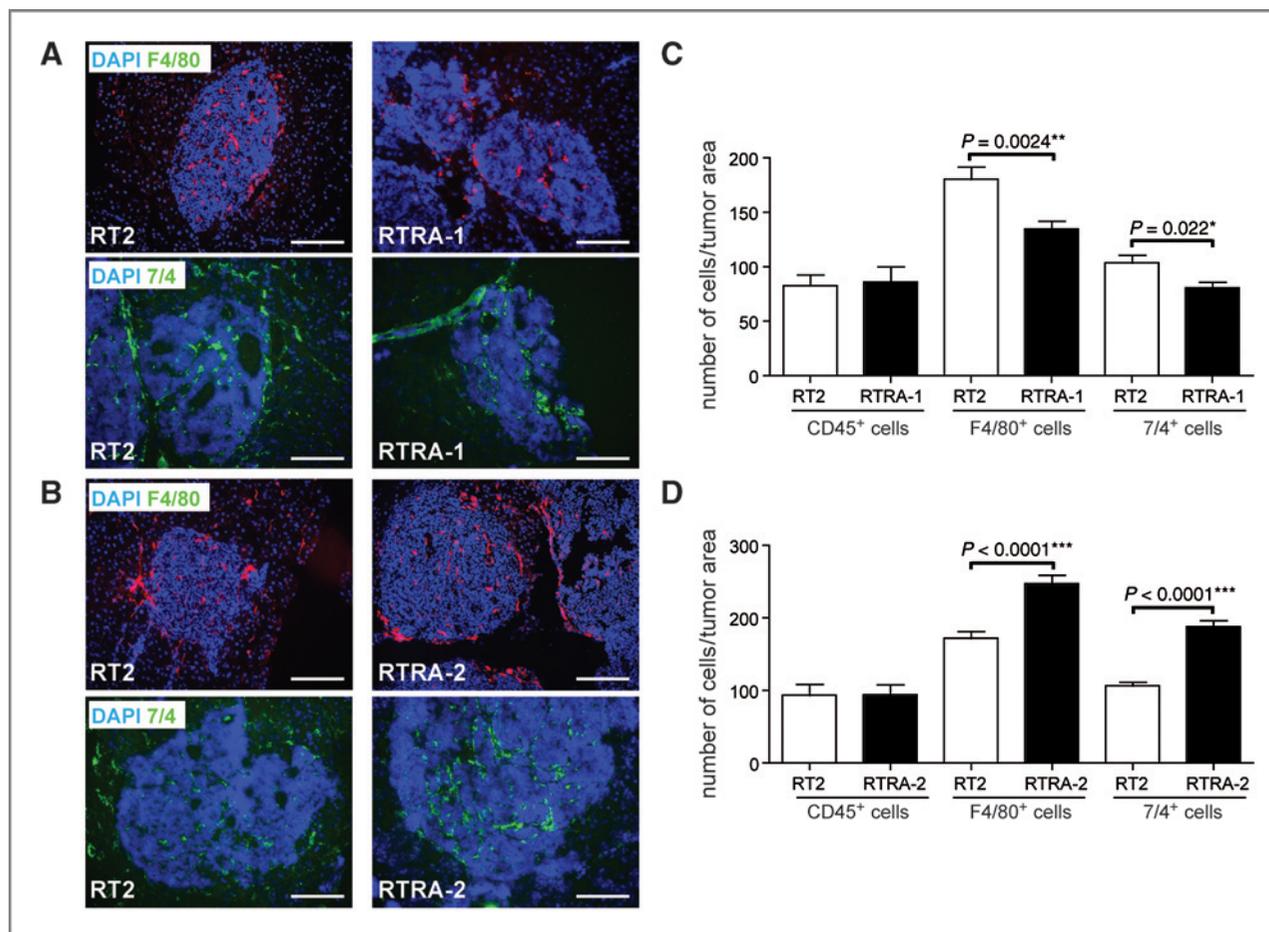


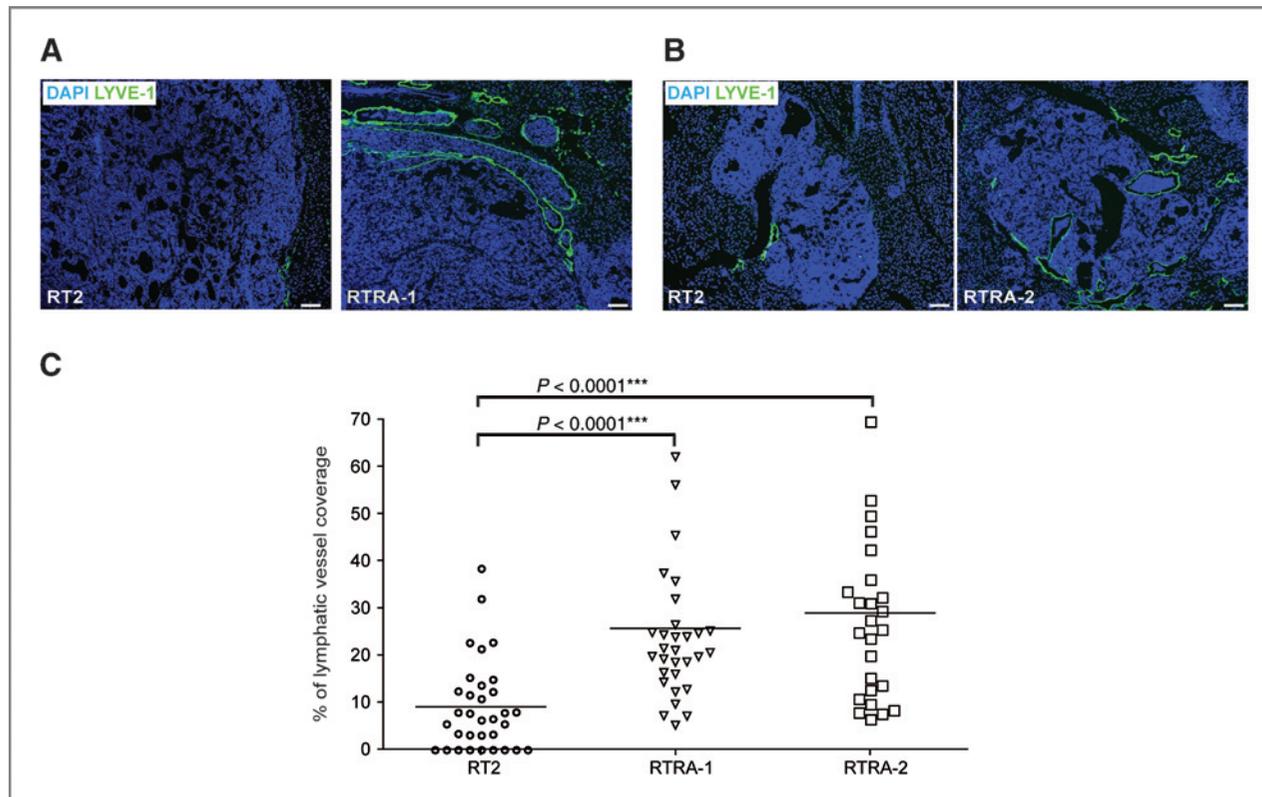
Figure 6. Tumor-infiltrating inflammatory cells in RTRA-1 and RTRA-2 mice. A and B, immunofluorescence analysis of tumor-infiltrating macrophages (F4/80<sup>+</sup> cells) and neutrophils (7/4<sup>+</sup> cells) on pancreatic sections from RT2 and RTRA-1 mice and from RT2 and RTRA-2 mice. Nuclei were visualized by staining with DAPI (blue). Bars, 100  $\mu$ m. C and D, quantification of tumor-infiltrated CD45<sup>+</sup> inflammatory cells, F4/80<sup>+</sup> macrophages, and 7/4<sup>+</sup> neutrophils in pancreatic tumors from RT2 ( $N = 3-5$ ,  $n = 11-57$ ), RTRA-1 ( $N = 3-5$ ,  $n = 8-43$ ) and RTRA-2 ( $N = 3-5$ ,  $n = 5-44$ ) mice.  $N$  = number of mice,  $n$  = number of microscopic fields (magnification  $\times 20$ ). \*,  $P = 0.022$ ; \*\*,  $P = 0.0024$ ; \*\*\*,  $P = 0.0001$ , all by unpaired Student  $t$  test.

### Ang-1 and Ang-2-mediated tumor lymphangiogenesis

We next determined the lymphatic vessel densities in tumors of RTRA-1 and RTRA-2 double-transgenic mice by LYVE-1 immunofluorescence staining. In agreement with previous reports, tumors of single-transgenic RT2 mice rarely exhibited peritumoral lymphatics (38, 39). In contrast, tumors of RTRA-1 and of RTRA-2 double-transgenic mice were partially surrounded by lymphatic vessels (Fig. 7A and B). The extent of peritumoral lymphangiogenesis was quantified by determining the extent by which the tumor perimeters were associated with LYVE-1-positive lymphatic vessels (Fig. 7C). The results indicate that both Ang-1 and Ang-2 are able to stimulate peritumoral and, more infrequently, intratumoral lymphangiogenesis. However, in contrast to the transgenic expression of VEGF-C and VEGF-D in RT2 mice (39, 40), the increased lymphangiogenesis in RTRA-1 and in RTRA-2 mice did not result in metastasis to local lymph nodes or to distant organs (Supplementary Fig. S7).

### Discussion

We have investigated the effects of transgenic expression of human Ang-1 or human Ang-2 in pancreatic  $\beta$  cells during islet development in RA-1 or RA-2 mice and during  $\beta$ -cell carcinogenesis in double-transgenic RTRA-1 or RTRA-2 mice. Expression of either Ang-1 or Ang-2 has no measurable effect on islet morphology and physiology. In double-transgenic RTRA-1 mice, the transgenic expression of Ang-1 in  $\beta$ -tumor cells exerts only marginal effects on angiogenesis and tumor outgrowth: As is the case for RT2 controls, the vessels are mature and well perfused, and only tumor cell proliferation is increased as compared with RT2 controls. In RT2, however, most of the blood vessels are already covered by pericytes. Thus, transgenic expression of Ang-1 may only have a redundant function on tumor angiogenesis, and the changes observed in increased pericyte-endothelial cell association, vessel functionality, and tumor growth are subtle and statistically not significant. These results are consistent with pre-



**Figure 7.** Ang-1 and Ang-2 induce peritumoral lymphangiogenesis. **A**, lymphatic vessels were visualized by immunofluorescence staining with anti-LYVE-1 antibodies (green) on pancreatic sections from RT2 and RTRA-1. Nuclei were stained with DAPI (blue). **B**, LYVE-1 (green) immunofluorescence staining of pancreatic sections from RT2 and RTRA-2 mice, as in (A). **C**, quantification of LYVE-1-positive lymphatic vessels on sections of tumors from RT2 ( $N = 4$ ,  $n = 33$ ), RTRA-1 ( $N = 4$ ,  $n = 31$ ), and RTRA-2 ( $N = 6$ ,  $n = 26$ ) mice. Results are presented as the percentages of lymphatic vessel coverage of individual tumors. \*\*\*,  $P < 0.0001$ , Mann-Whitney test.  $N$  = number of analyzed mice,  $n$  = number of analyzed individual tumors. Bars, 100  $\mu$ m.

vious reports showing that Ang-1 recruits pericytes to blood vessels and leads to an expanded and stabilized, leakage-resistant vasculature (9, 41, 42).

In contrast, in double-transgenic RTRA-2 mice reduced tumor cell proliferation and tumor growth and increased tumor cell apoptosis is observed. Although endothelial cell density is increased in RTRA-2 tumors, vessels are highly disorganized, lack pericyte coverage, and are poorly perfused, indicating that endothelial cell density may not give a direct measure of tumor perfusion and tumor growth. These results are in agreement with previous reports showing that Ang-2 expression in xenografted or endogenously growing tumors inversely correlates with pericyte coverage (43, 44) or leads to massive tumor vessel regression (45).

In RTRA-1 mice, the number of tumor-infiltrating macrophages and neutrophils is found reduced. This could be due to the inhibitory action of Ang-1 on endothelial gap formation and plasma leakage (46). Ang-1 also exerts anti-inflammatory activities, protects against cardiac allograft arteriosclerosis and radiation-induced endothelial cell damage, and promotes wound healing (5, 6). Furthermore, Ang-1 can repress VEGF-induced blood vessel formation and adhesion-molecule expression.

In contrast, in RTRA-2 mice the numbers of tumor-infiltrating macrophages and neutrophils are significantly

increased. Ang-2 is known to promote inflammatory responses by activating the endothelium and inducing vessel permeability (5, 6). Tumor-infiltrating inflammatory cells, such as macrophages, neutrophils, and mast cells, have been repeatedly shown to play a critical role in the angiogenic switch during tumorigenesis by providing additional angiogenic growth factors, including VEGF-A, and MMPs that activate matrix-associated VEGF-A (2). The elevated levels of VEGF-A (and of other angiogenic factors) lead to overshooting, nonproductive tumor angiogenesis with an increased density of nonfunctional, immature vessels. VEGF-A is critically involved in the angiogenic switch during tumor progression in RT2 mice, as previously shown by the conditional ablation of VEGF-A expression or adenoviral delivery of soluble VEGF receptors which efficiently blocked tumor angiogenesis and tumor outgrowth (22, 26, 47). Besides, VEGF induces proteolytic shedding of the extracellular domain of Tie-2 into a 75-kDa soluble fragment (sTie-2) able to bind Ang-1 and to block Tie-2 activation and vessel maturation (48).

Consistent with the notion of increased inflammatory cell infiltration and VEGF-A expression in tumors of RTRA-2 mice, tumor vessel regression, and tumor growth arrest are more pronounced when RTRA-2 mice are treated with the VEGF receptor inhibitor PTK/ZK. We conclude that Ang-2 and

VEGF act together to induce tumor angiogenesis in RTRA-2 transgenic mice. Hence, Ang-2 exerts a dual function: In conjunction with VEGF-A it exerts a proangiogenic activity by promoting vessel sprouting and growth, whereas it destabilizes newly formed blood vessels by preventing mural cell recruitment, vessel maturation, and efficient perfusion. As a result of nonfunctional angiogenesis and impaired vessel perfusion, tumor growth is retarded in RTRA-2 mice. Conversely, it has been reported that repression of Ang-2 activity leads to an inhibition of VEGF-A-mediated angiogenesis and of tumor growth (49).

These findings support a model that a balanced Ang-1:Ang-2 ratio determines the functional status of the vasculature. In RTRA-1 mice, vessel maturation and vessel functionality are maintained by constitutive Ang-1/Tie-2 signaling. The functionality of the vessels ensures appropriate tumor perfusion, high tumor cell proliferation, and low apoptosis. In RTRA-2 mice, high levels of Ang-2 destabilize blood vessels and impair their functionality. Yet, on the other hand, Ang-2 primes blood vessels to respond to other cytokines, such as VEGF-A, which induce an inflammatory and angiogenic response, as observed by the increased recruitment of inflammatory cells to the tumor microenvironment.

Our experiments have also revealed a lymphangiogenic activity for both Ang-1 and Ang-2. Peri-insular and peritumoral lymphangiogenesis was significantly increased in RA-1 or RA-2 and in RTRA-1 or RTRA-2 transgenic mice. However, the increased lymphangiogenesis in RTRA-1 or RTRA-2 did not provoke the formation of metastasis to regional lymph nodes or distant organs, as has been observed by the transgenic expression of VEGF-C or

VEGF-D in RT2 mice (39, 40). Previously, it has been reported that Ang-1 enhances *in vitro* colony formation of lymphatic endothelial cells and induces *in vivo* lymphatic vessel formation in the mouse cornea (30). Conversely, Ang-2-deficient mice exhibit defects in the development and function of the lymphatic vasculature, which can be completely rescued by Ang-1 (31). Thus, Ang-1 and Ang-2 seem to exert similar functions as lymphangiogenic factors during lymphangiogenesis and in lymphatic vessel homeostasis. Whether these functions are exerted via binding exclusively to Tie-2 receptor or to Tie-1/Tie-2 heterodimers or even to other coreceptors warrants further investigation.

### Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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