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Inhibition of VEGF-Dependent Multistage Carcinogenesis by Soluble EphA Receptors[1]

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Abstract

Elevated expression of Eph receptors has long been correlated with the growth of solid tumors. However, the functional role of this family of receptor tyrosine kinases in carcinogenesis and tumor angiogenesis has not been well characterized. Here we report that soluble EphA receptors inhibit tumor angiogenesis and tumor progression *in vivo* in the RIP-Tag transgenic model of vascular endothelial growth factor (VEGF)–dependent multistage pancreatic islet cell carcinoma. Soluble EphA receptors delivered either by a transgene or an osmotic minipump inhibited the formation of angiogenic islet, a premalignant lesion, and reduced tumor volume of solid islet cell carcinoma. EphA2-Fc or EphA3-Fc treatment resulted in decreased tumor volume but increased tumor and endothelial cell apoptosis *in vivo*. In addition, soluble EphA receptors inhibited VEGF and βTC tumor cell–conditioned medium-induced endothelial cell migration *in vitro* and VEGF-induced cornea angiogenesis *in vivo*. A dominant negative EphA2 mutant inhibited—whereas a gain-of-function EphA2 mutant enhanced—tumor cell–induced endothelial cell migration, suggesting that EphA2 receptor activation is required for tumor cell–endothelial cell interaction. These data provide functional evidence for EphA class receptor regulation of VEGF-dependent tumor angiogenesis, suggesting that the EphA signaling pathway may represent an attractive novel target for antiangiogenic therapy in cancer.

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Introduction

Traditional preclinical studies testing the efficacy of a compound in a tumor model usually involve subcutaneous or intravenous injection of human tumor cells into immunodeficient mice. Although these "proof-of-principle" experiments provide valuable information in an *in vivo* system, recent clinical trials of antiangiogenic agents, such as matrix metalloproteinase (MMP) inhibitors, revealed that these ectopic graft models are inadequate and do not recapitulate host–tumor interactions [1,2]. Thus, it is critical to evaluate antiangiogenic agents in an animal model where tumors arise from normal cells in their natural tissue microenvironments and progress through multiple stages, as does human cancer.

One attractive target for antiangiogenic cancer therapy is the Eph family of receptor tyrosine kinases. The Eph family of RTKs and their ligands, known as ephrins, directs a variety of biologic processes during embryogenesis [3–6]. The role of this family of RTKs in cancer is complex and has just begun to be investigated (reviewed in Refs. [5,7]). Aside from a potential role in tumor cells, recent studies suggest that class A Eph RTKs are also essential in regulating tumor angiogenesis. First, a survey of expression patterns of Eph molecules in tumor vasculature revealed that the ephrin-A1 and EphA2 ligand–receptor pair is consistently expressed in endothelial cells of tumor-associated vessels in a variety of tumors, including tumor xenografs (MDA-MB-435 human breast cancer and KS1767 human Kaposi's sarcoma) and human tumor specimens (breast cancer, lung anaplastic adenocarcinoma, squamous carcinoma, gastric cancer, colon carcinoma, and kidney clear cell carcinoma) [8]. Second, ephrin-A1 and EphA2 were also found to be expressed in tumor endothelial cells in two murine models that are angiogenesis-dependent: the RIP-Tag islet carcinoma model and 4T1 transplantable metastatic

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mammary carcinoma model [9]. Finally, we have shown that local administration of soluble EphA2-Fc chimeric receptors could inhibit tumor growth and angiogenesis in a subcutaneous graft of 4T1 mammary carcinoma cells, a model that is involved in transplantation of malignant tumor or tumor cells into an ectopic site in a recipient mouse [9].

Although the above studies support a role of class A Eph molecules in tumor progression, the functional relevance of the above studies to tumors spontaneously arisen in an endogenous environment remains to be determined. In this report, we studied the effect of soluble EphA2 and EphA3 receptor Fc fusion proteins (EphA2-Fc/EphA3-Fc) on pancreatic islet cell carcinoma in the RIP-Tag transgenic mice. In this model, tumors arise spontaneously in an endogenous environment and the development of tumors involves multiple stages, progressing from morphologically normal islets, hyperplasia, angiogenic dysplasia, to invasive islet cell carcinoma (reviewed in Refs. [10–12]). Here we report that local or systemic delivery of soluble EphA receptors significantly inhibits the formation of angiogenic islets and reduces tumor volume. Furthermore, studies of interactions between RIP-Tag tumor cells and endothelial cells *in vitro* indicate that EphA2 receptor and vascular endothelial growth factor (VEGF) signaling pathways are required for endothelial cell activation by tumor cells. Thus, blockade of EphA signaling inhibits tumor-induced endothelial cell migration and represents a testable therapeutic approach against cancer.

Materials and Methods

Soluble EphA-Fc Receptors

The EphA2-Fc soluble receptor cDNA construct containing the extracellular domain of EphA2 (a.a. 1–495) and Fc portion of IgG1 was provided by Dr. N. Gale (Regeneron, Tarrytown, NY) and subcloned into episomal expression vector pCEP4 (Invitrogen, San Diego, CA). Recombinant EphA2-Fc proteins were either purified from a culture supernatant of stable 293T clones expressing these factors using protein A Sepharose column, or purchased from R&D Systems (Minneapolis, MN). Recombinant EphA3-Fc protein was provided by Immunex (Seattle, WA). To inhibit binding to Fc receptors and complements, the following amino acids in human IgG1 lambda chain Fc region were mutated in the EphA3-Fc construct: L264A, L265E, and G267A.

Mouse Strains and Maintenance

RIP1-Tag2 C57BL/6 mice (generously provided by Dr. Doug Hanahan, University of California at San Francisco) and wild-type C57BL/6 mice were maintained in accordance with AAALAC and Vanderbilt University guidelines. RIP1-Tag2 transgenic animals were maintained on a sucrose-enriched diet (Teklad Test Diets, Madison, WI) and 4% glucose water. Animals positive for the RIP-Tag transgene were identified by polymerase chain reaction (PCR) analysis of genomic DNA from tail biopsy using the following primers: 5′-GGACAAACCACAACTAGAATG-3′ and 5′-CAGAGCAGAATTGTGGAGTGG-3′.

*Eco*RI–*Not*I fragment containing EphA2-Fc chimeric fusion cDNA (provided by Dr. Nick Gale; Regeneron) was blunted and subcloned into the *Cla*I site of transgenic RIP-7 vector (provided by Dr. Hanahan, University of California at San Francisco). The linearized fragment encompassing the tissue-specific transcription unit was microinjected into the pronuclei of C57BL/6×DBA/2 zygotes. Transgenic founders were identified by genomic Southern blot and PCR analyses using the following primers: 5′-ACGCTGACCACACGGTACGT-3′ and 5′-AGCCCCACATGAACTATACC-3′. RIP-EphA2-Fc transgenic mice were backcrossed to C57BL/6 for three generations before intercrossing with RIP-Tag mice.

Pharmacologic Intervention

RIP1-Tag2 transgenic mice on C57BL/6 background were used in these studies. Animals were treated either from 8 to 10 weeks of age, or from 10 to 12 weeks of age. Five hundred micrograms of soluble EphA2-Fc, EphA3-Fc, or Fc control (IgG) was loaded into 2-week osmotic pumps (model 2002; Alzet, Osmotic Pumps, Cupertino, CA), which were implanted intraperitoneally. Animals were monitored three times a week and sera were collected for enzyme-linked immunosorbent assay (ELISA) to determine the serum concentration of Fc proteins.

Assessment of Angiogenic Islets and Tumor Volume

Pancreatic islets were isolated by retrograde perfusion with collagenase P as described [13]. Angiogenic islets were identified as those that exhibited a reddish patch in a white nodular background and enumerated under the dissecting microscope. The visual scoring was confirmed by histology. Tumors were microdissected from freshly excised pancreases. Tumors (>1 mm in diameter) were measured with calipers, and tumor volumes were calculated as described [volume = $0.52\times(\text{width})^2\times$ length] [13].

ELISA for Fc Proteins

Serum samples were collected before osmotic pump implantation and 4, 7, 10, and 14 days after implantation for the detection of EphA2-Fc, EphA3-Fc, and control IgG levels. In brief, ELISA plates were coated overnight at 4°C with serum samples in serial dilutions (1:20, 1:40, and 1:60) in coating buffer (34.4 mM sodium bicarbonate, 15 mM sodium carbonate, and 3 mM sodium azide). Standards were prepared by diluting IgG protein (Sigma, St. Louis, MO) with coating buffer (from 0.01 to 100 ng/ml). After washing with PBS containing 0.1% Tween 20, nonspecific binding was blocked by PBS containing 10% FBS for 1 hour at room temperature. Samples were then incubated with affinity-purified biotinylated goat antihuman IgG (1 μg/ml) antibodies (crossreactivity with mouse IgG <1%; Vector Laboratories, Burlingame, CA) at room temperature for 2 hours, followed by incubation with avidin conjugated alkaline phosphatase (1:400 dilution; Sigma) for 30 min. Plates were developed using TMB as substrates (Sigma). The color reaction was stopped by 1 M HCl and the OD was measured at 450 nm using a microplate reader and Delta3 software.

Histologic Analyses

Pancreatic tissue was fixed in 10% neutral-buffered formalin (Fisher) overnight at 4°C, subjected to graded dehydration and paraffin embedding, and 7-μm sections were prepared for immunohistochemistry. For Fc protein staining, samples were blocked for 1 h in PBS containing 5% sheep

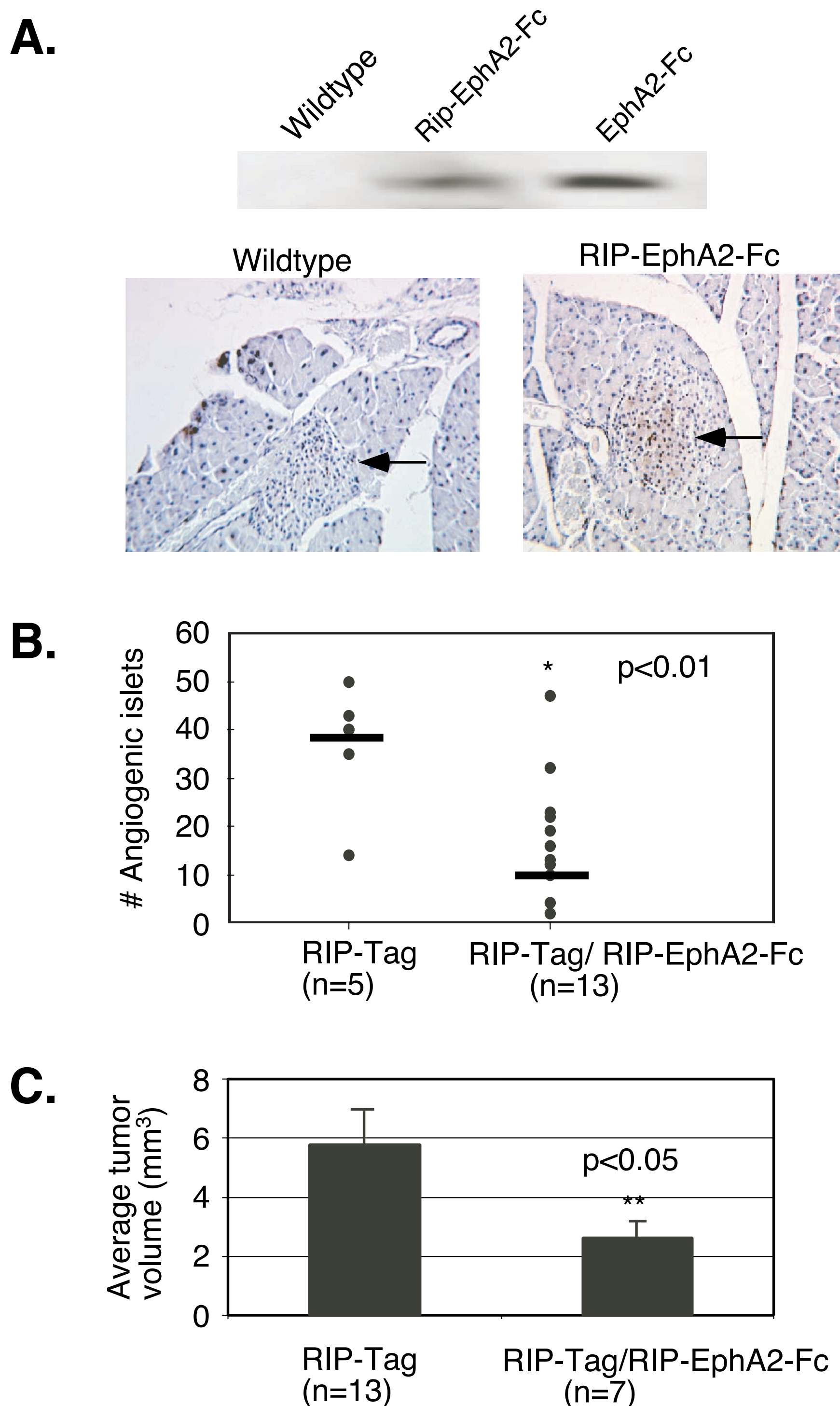


Figure 1. *Inhibition of angiogenic islet formation and tumor volume in RIP-EphA2-Fc/RIP-Tag transgenic mice. (A) RIP-EphA2-Fc transgenic mice express soluble EphA2-Fc in pancreatic islets. Total pancreatic lysates from either transgenic RIP-EphA2-Fc mice or control wild-type littermates were immunoprecipitated by protein A/G beads and subjected to Western blot analysis using a goat antihuman IgG antibody (top panel). Islet-specific expression of soluble EphA2-Fc was confirmed by immunohistochemistry (bottom panel). EphA2-Fc: purified recombinant protein used as positive control. Arrows indicate pancreatic islets. (B) Inhibition of angiogenic islet formation in RIP-EphA2-Fc/RIP-Tag double transgenic mice. Pancreatic islets were isolated from 10-week–old RIP-EphA2-Fc/RIP-Tag double transgenic mice or wild-type control littermates by retrograde perfusion with collagenase P. Angiogenic islets were identified as those exhibiting a reddish patch in a white nodular background and counted. (C) Inhibition of tumor volume in RIP-EphA2-Fc/RIP-Tag double transgenic mice. Pancreatic islet tumors were microdissected from freshly excised pancreases from 12-week–old mice. Tumors (>1 mm in diameter) were measured with calipers and tumor volumes were calculated as described in Materials and Methods section. Statistical analysis was performed by two-tailed, unpaired, Student's t-test. *P < .01; **P < .05 vs control.*

serum (Sigma) and incubated with affinity purified biotinylated goat antihuman IgG antibodies (1:100 dilution; Vector Laboratories) overnight at 4°C. For VEGFR2 (Flk-1)/TUNEL immunofluorescence double staining, sections were pretreated with proteinase K (Dako, Cytomation, Denmark) for 10 minutes at 37°C, blocked with PBS/5% sheep serum for 1 hour, and incubated with polyclonal rabbit anti-VEGFR antibodies T014 (5 μg/ml) (kindly provided by Dr. R. Brekken, UT Southwest) overnight, followed by secondary biotinylated anti-rabbit IgG (Pharmingen, San Diego, CA) and streptavidin-conjugated Alexa 488 flourochrome (Molecular Probes, Eugene, OR). Sections were then subjected to TUNEL assay using an ApopTag Red *in situ* apoptosis detection kit (Intergen, Purchase, NY) and counterstained with DAPI (0.05 μg/ml in PBS; Sigma) to visualize nuclei. Apoptotic cells were quantified by numerating rhodamine-stained nuclei. Percentage of total apoptosis was calculated as the number of rhodamine-stained nuclei/total number of DAPI-stained nuclei, using Scion Image software. Apoptotic endothelial cells were determined by cells stained positive for both TUNEL (red) and VEGFR (green) (double positive cells, yellow in overlay image). Percentage of apoptotic cells was then calculated as the number of double positive cells/total number of DAPI-stained nuclei. Experiments were repeated three times and results were pooled. Statistical analysis was performed using the two-tailed, paired Student's *t*-test.

Endothelial and βTC Cell Culture

The β islet carcinoma cell line (βTC) derived from RIP-Tag mice (provided by Dr. Hanahan) were maintained in DMEM/30% fetal bovine serum. Human primary umbilical endothelial cells (HUVECs) and bovine primary pulmonary microvascular endothelial cells (BPMECs) were purchased from Clonetics (Walkersville, MD) and Vec Technologies (Rensselaer, NY), respectively, and maintained in 10-cm dishes coated with 0.1% gelatin in complete EBM medium (Clonetics). Cells were used up to six passages. For tumor-conditioned medium, βTCs were grown to 80% confluency, washed with PBS, and incubated in minimal volume (3 ml for a 10-cm dish) of low serum medium (DMEM/2% FBS) overnight. Conditioned medium was collected after 24 hours of incubation.

Migration Assays

Endothelial cell migration was determined by wound closure assay as described previously [14]. Briefly, replicate circular "wounds" were generated in confluent HUVEC cell monolayers using a silicon-tipped drill press. Endothelial cells were then treated with tumor cell–conditioned medium in the presence or absence of 5 μg/ml EphA2-Fc or 10 μg/ml neutralizing antibodies against VEGF. Residual fractional "wound" areas were measured following 0, 4, 8, and 12 hours using Bioquant software. A "wound" completely closed is referred to as 100% closure. Experiments were repeated three times and results were pooled. Statistical analysis was performed by Student's *t*-test.

Mouse Corneal Angiogenesis Assay

Hydron pellets incorporating sucralfate with recombinant human VEGF (160 ng/pellet; R&D Systems) were made as described [15]. Pellets were surgically implanted into corneal stromal micropockets created 1 mm medial to the lateral corneal limbus of C57L male mice (7 weeks old). The mice were treated systemically by daily intraperitoneal injection of 400 μg of IgG1 control (Sigma) or EphA3-Fc (Immunex), beginning 1 day prior to implantation. At day 5, corneal angiogenic responses were photographed using an Insight Spot digital camera under Leico dissection microscope. The percentage of vascularized area/total corneal image was calculated using the Spot software. Each value is the mean±SEM of six corneas for each condition. Statistical analysis was performed using the two-tailed, paired Student's *t*-test ($P < .05$).

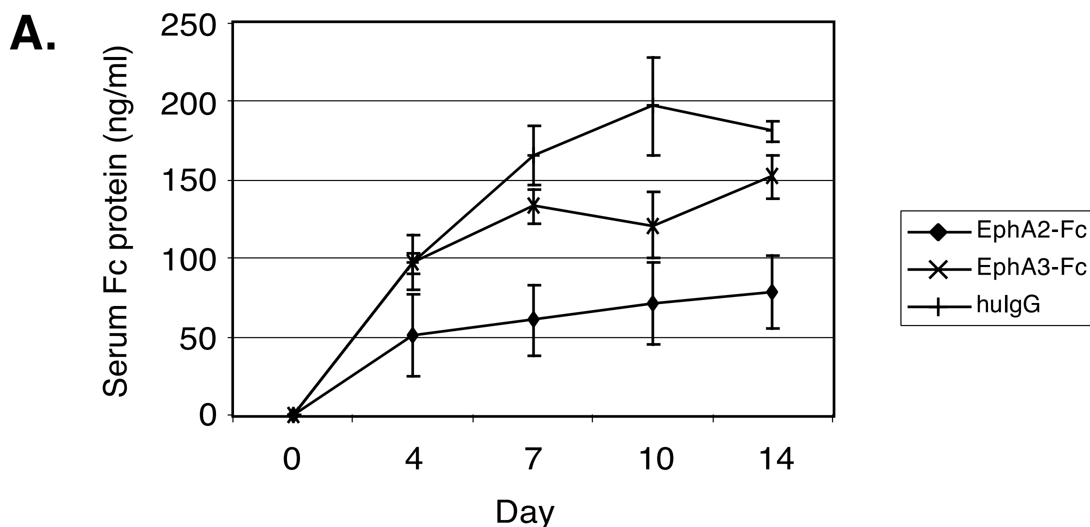
Co-culture Migration Assay

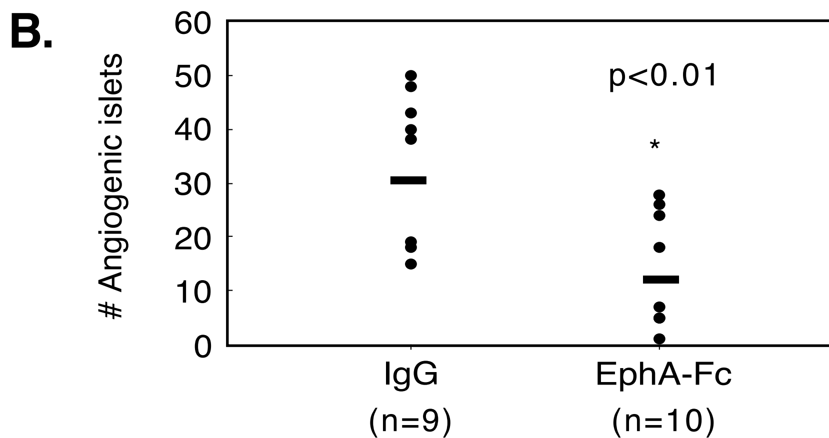
EphA2-Neu was generated by substituting the transmembrane domain (a.a. 496–516) with that from the oncogenic neu (VTFIIATVEGVLLFLILVVVVGILI) [16]. The myc-tagged dominant negative EphA2 cytoplasmic domain truncation mutant (EphA2-ΔC) was generated by PCR amplification of the extracellular and transmembrane domains of EphA2 (a.a. 1–521). COS-1 cells were transfected with 10 μg of pECE-EphA2WT or pECE-EphA2Neu, or mock-transfected using Lipofectin. EphA2 was immunoprecipitated and subjected to SDS-PAGE and Western blotting using an anti-phosphotyrosine (PY) antibody. To test cytoplasmic domain truncation mutant of EphA2, COS-7 cells were transiently transfected with 10 μg of pcDNA3-EphA2-ΔC or empty vector pcDNA3 by DEAE dextran method. Two days after transfection, cells were starved in DMEM without serum followed by stimulation of 1 μg/ml ephrin-A1 for 15 minutes. Endogenous EphA2 receptor was immunoprecipitated and subjected to SDS-PAGE and Western blotting using anti-PY antibodies (pY99 and pY20; Santa Cruz Biotechnology,

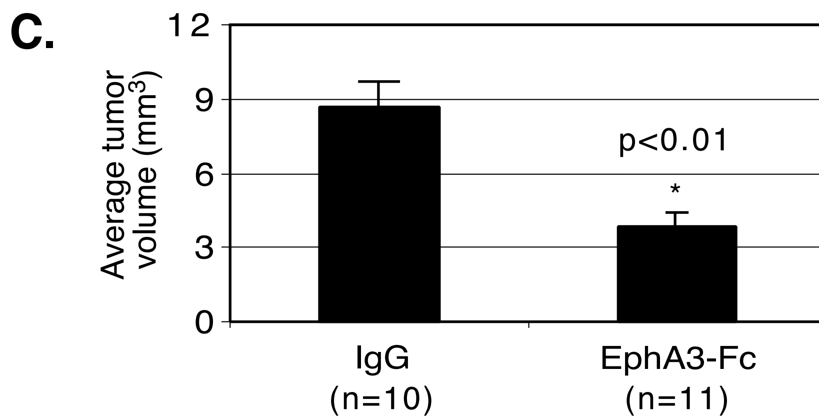
Figure 2. *Osmotic minipump delivery of soluble EphA receptors inhibits angiogenic islet formation and tumor volume in RIP-Tag mice. (A) Serum concentration of Fc proteins. Serum samples were collected before osmotic pump implantation and 4, 7, 10, and 14 days postimplantation for the detection of soluble EphA2-Fc (—◆—), EphA3-Fc (—×—), and control IgG levels (—+—) in the circulation. Serum concentration of these Fc chimeric proteins was determined by ELISA using a goat antihuman IgG antibody. (B) Inhibition of angiogenic islet formation by 2-week treatment of soluble EphA receptors. An osmotic minipump containing 500 μg of soluble EphA receptor or Fc control protein, IgG, was implanted intraperitoneally in RIP-Tag mice at 8 weeks of age. Angiogenic islets were isolated from treated or control mice at 10 weeks of age and enumerated. EphA-Fc, EphA2-Fc, or EphA3-Fc. (C) Soluble EphA receptors inhibited tumor volume in RIP-Tag mice. An osmotic minipump containing 500 μg of soluble EphA receptor or Fc control protein, IgG, was implanted intraperitoneally in RIP-Tag mice at 10 weeks of age. Islet tumors were isolated from treated or control mice at 12 weeks of age and tumor volumes were calculated. (D) Soluble EphA receptor inhibited tyrosine phosphorylation of endogenous EphA2 receptor in islet tumor. Endogenous EphA2 from IgG or soluble EphA receptor–treated tumors (1 mg of protein lysates) were immunoprecipitated by an anti-EphA2 antibody and subjected to Western blot analysis using antiphosphorylated tyrosine antibodies. Statistical analysis was performed by two-tailed, unpaired, Student's t-test. *P < .01 vs control.*

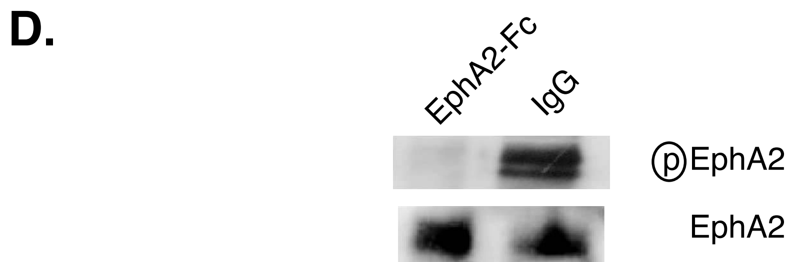
Santa Cruz, CA). Blots were stripped and reprobed for EphA2 expression to ensure equal loading (D7; Upstate Biotechnology, Lake Placid, NY).

Prior to co-culture, 0.5×10^6 bovine pulmonary microvascular endothelial cells (BPMECs) were seeded in 6 well dishes and transfected with 1 μg of pGABE control









GFP vector (Mock), wild-type EphA2 (WT), EphA2-ΔC (ΔC), or EphA2-Neu (Neu) using the Lipofectamine 2000 reagent. Transfection efficiency was estimated to be approximately 50%, judging by GFP expression in control plates. Overexpression of EphA2-Neu and EphA2-ΔC mutants was confirmed by immunoblot analysis using anti-EphA2 (Santa Cruz Biotechnology or Upstate Biotechnology) and anti-myc (Pharmingen) antibodies, respectively. For activity assay, BPMECs expressing wild-type EphA2, EphA2-Neu, or EphA2-ΔC were stimulated with 1 μg/ml soluble ephrin-A1-Fc, EphA2-immunoprecipitated, and subjected to PY blot analysis.

BPMEC/βTC coculture migration assays were adapted from a modified Boyden chamber assay and performed as described [9]. Briefly, βTCs were labeled with FITC ovalbumin and grown on the underside of the filter (pore size 8 μm). BPMECs labeled with Texas Red ovalbumin were added to upper transwell chambers. After 5 hours, cells were removed from the upper surface of the transwell filter using a cotton swab, and endothelial cell density on the lower surface of the filter was quantified by counting red fluorescent pixels using Scion Image software analysis. Data are representations of eight independent samples per condition with standard deviation, and statistical significance was determined by two-tailed, paired Student's *t*-test.

Results

Inhibition of Tumor Angiogenesis and Tumor Growth in RIP-EphA2-Fc/RIP-Tag Double Transgenic Mice

To investigate the role of class A Eph receptors and ephrin ligands in multistage carcinogenesis *in vivo*, we utilized the RIP-Tag transgenic mouse model of pancreatic islet cell carcinoma, in which the rat insulin promoter drives expression of the SV40 large T antigen oncogene in the β-cells of pancreatic islets [10–12]. These animals undergo tumorigenesis in a reproducible series of stages, including hyperplasia, angiogenic dysplasia, and invasive carcinoma, one of which involves the initiation of angiogenesis. Ephrin-A1 ligand is overexpressed in RIP-Tag islet cell carcinomas, both in tumor and endothelial cells, whereas EphA2 receptor is expressed in tumor-associated vasculature, suggesting a proangiogenic function for this ligand–receptor pair [9]. As a first step to determine whether blockade of EphA receptor function could affect multistage tumorigenesis, we generated RIP-EphA2-Fc transgenic mice to deliver soluble EphA2 receptor locally in the pancreatic islet, using the same rat insulin promoter as in RIP-Tag mice. As shown in Figure 1*A*, EphA2-Fc proteins were expressed in the pancreatic islet in transgenic mice, as judged by Western blot analysis and immunohistochemistry. The RIP-EphA2-Fc transgenic mice developed and bred normally in the pathogen-free animal facility and there were no significant changes in the number and morphology of pancreatic islets (data not shown) between RIP-EphA2-Fc transgenic animals and control non-transgenic littermates, suggesting that soluble EphA receptor does not affect normal islet development.

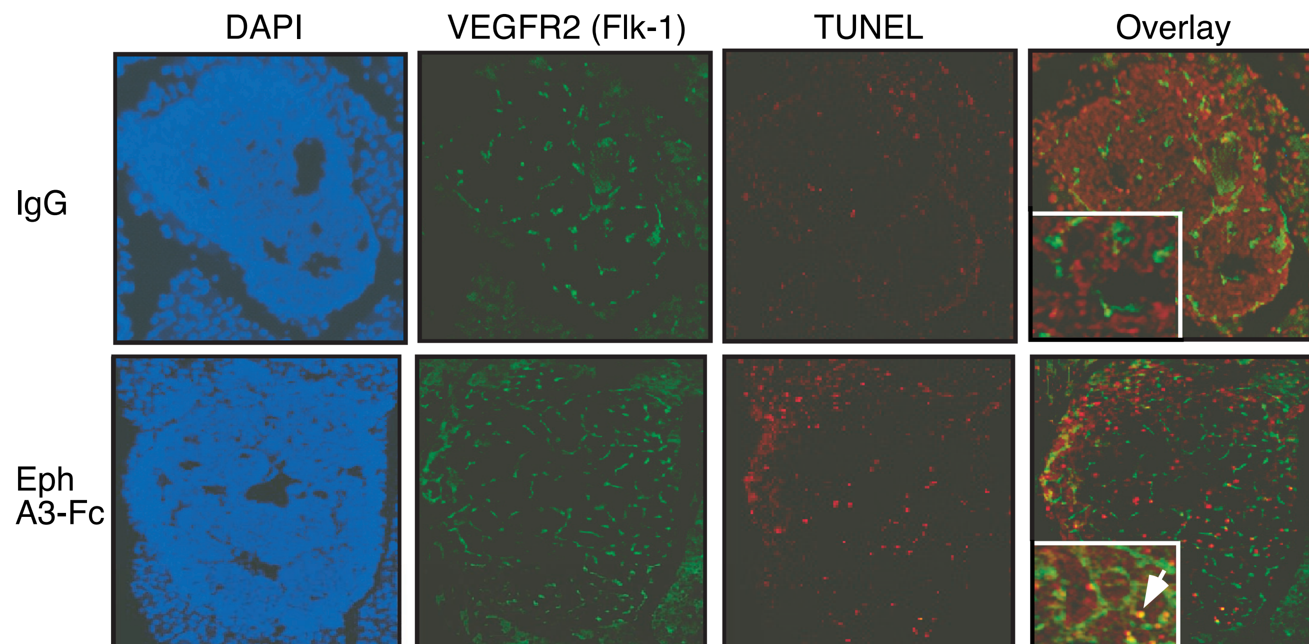
The RIP-EphA2-Fc transgenic mice were backcrossed into C57BL/6 background for three generations, and intercrossed with RIP-Tag transgenic mice to determine the effect of soluble EphA2 receptor on tumor progression *in vivo*. In the RIP-Tag model, islets expressing SV40 Tag oncogene are morphologically normal until about 4 weeks of age. Hyperplastic islets then begin to develop as white nodules with hyperproliferation and characteristics of carcinoma *in situ*. Angiogenic islets arise from hyperplastic nodules at approximately 8 weeks of age as white nodules with reddish patches, characterized by endothelial sprouting, microhemorrhaging, and the ability to elicit an angiogenic response *in vitro*. Highly vascularized small solid tumors emerge at 10 weeks and progress to merge into a few, large, invasive carcinomas (12–14 weeks).

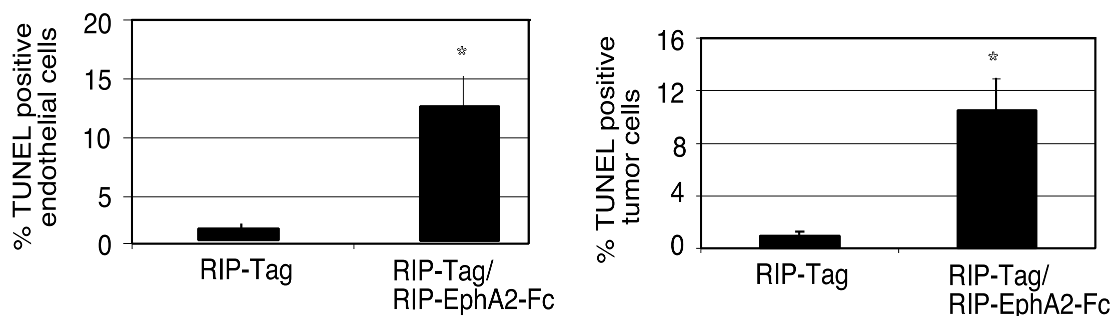
Two specific stages of tumorigenesis were examined in RIP-Tag mice: the formation of angiogenic islets/small tumors at 10 weeks of age, and tumor volume of highly angiogenic islet cell carcinoma at 12 weeks of age. As shown in Figure 1*B*, at 10 weeks of age, there is two-fold decrease in the number of angiogenic islets and small tumors in RIP-EphA2-Fc/RIP-Tag double transgenic mice, compared to RIP-Tag single transgenic littermate controls. By 12 weeks of age, control RIP-Tag mice have developed several large tumors. In contrast, the tumors in the RIP-Tag/RIP-EphA2-Fc double transgenic mice are much smaller, with a two-fold reduction in average tumor volume (Figure 1*C*). The numbers of tumors in dual RIP-Tag/RIP-EphA2-Fc and in control RIP-Tag mice did not vary significantly (7.2 ± 2.5 in RIP-Tag controls compared to 8.6 ± 1.8 in dual transgenic mice). Thus, the phenotypes in double transgenic mice at 12 weeks resemble those at 10 to 11 weeks in RIP-Tag control mice, reflecting the inhibition of small tumor growth and merging into large tumors by soluble EphA2-Fc receptors. There is no significant morphologic change in the tumor section, except that apoptosis levels were increased in tumors from dual transgenic mice (Figure 3). Taken together, these data suggest that soluble EphA receptors inhibit angiogenic islet formation and small tumor progression in large tumors, although they do not block tumor formation entirely.

Osmotic Pump Delivery of Soluble EphA Receptors Inhibited Formation of Angiogenic Premalignant Lesions and Islet Carcinoma Progression in RIP-Tag Mice

Encouraged by the results in RIP-EphA2-Fc/RIP-Tag double transgenic mice, we designed a study to assess the effects of exogenously delivered soluble EphA receptors in RIP-Tag mice at two distinct stages of pancreatic islet carcinogenesis. Two different forms of EphA soluble receptors, EphA2-Fc and EphA3-Fc, were used because mutations in the Fc portion of the EphA3-Fc chimeric protein block interaction with Fc receptors and complement [24–26]. Both EphA2-Fc and EphA3-Fc inhibited endogenous endothelial EphA receptor activation in response to ephrin stimulation and ephrin-A1–induced corneal angiogenesis (data not shown). An osmotic minipump was used to deliver 500 μg of soluble receptors or Fc control protein (IgG) over a 2-week period, as prior studies showed that in

comparison to bolus intraperitoneal injection, continuous administration of angiogenesis inhibitors by an osmotic pump implanted intraperitoneally resulted in a decrease of the required dosage and improved the effectiveness of therapy [17]. As shown in Figure 2*A*, soluble EphA receptors administered continuously through mini-osmotic pump





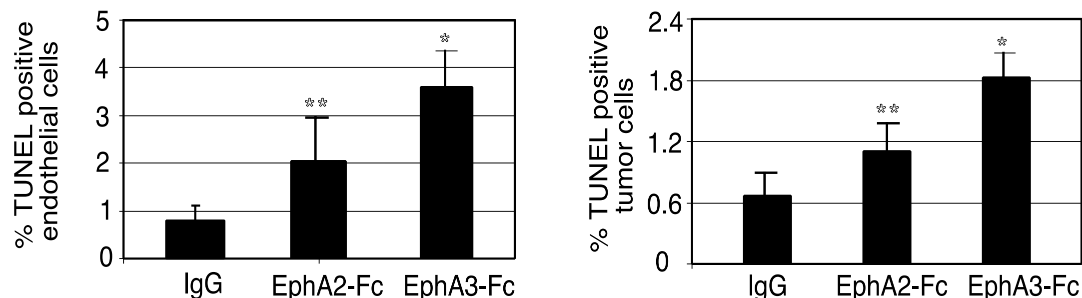
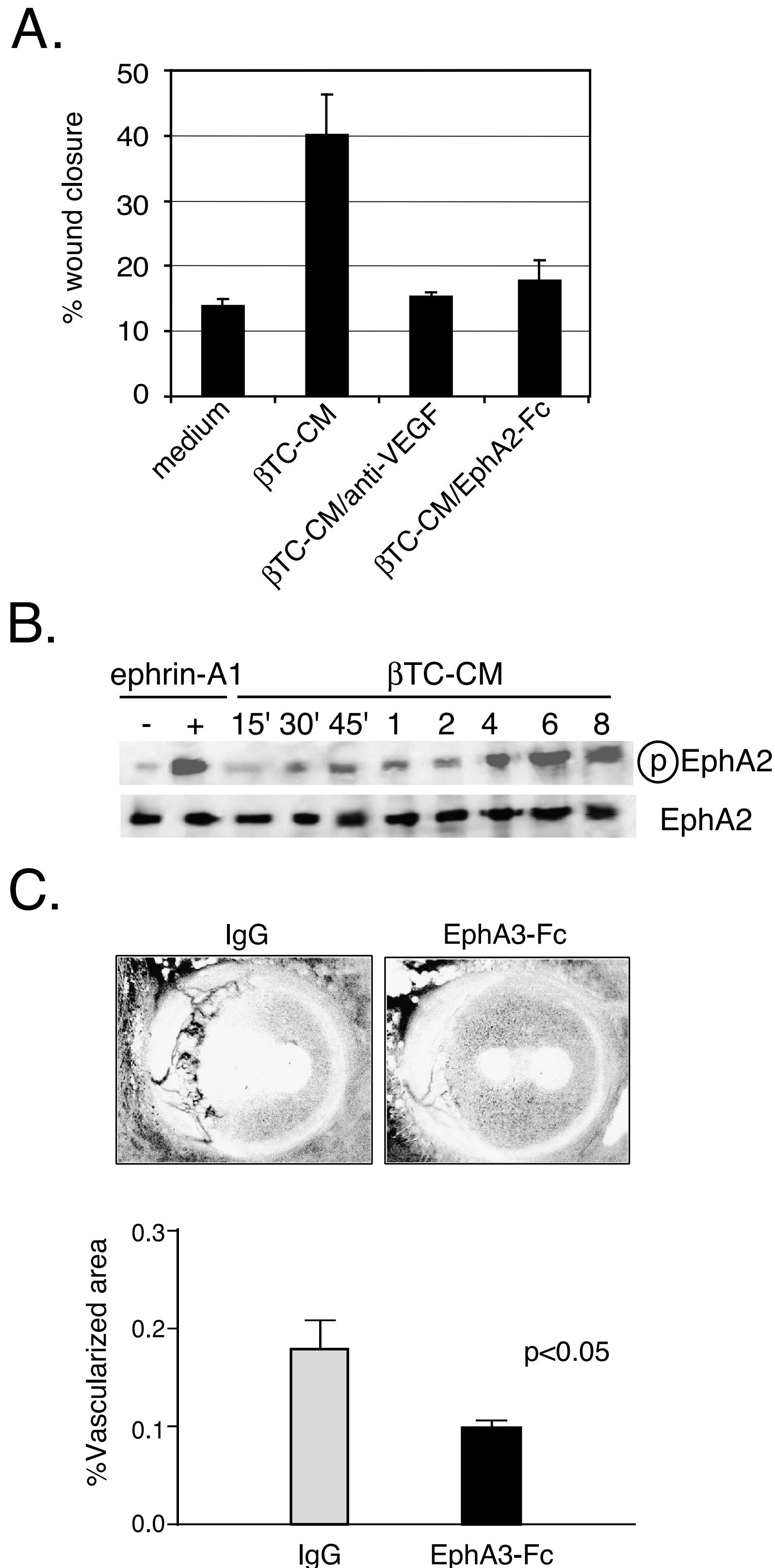


Figure 3. *Soluble EphA-Fc receptors enhance tumor cell and endothelial cell apoptosis in RIP-Tag islet tumors. Apoptosis in RIP-Tag tumors was assessed by dual immunofluorescence staining for VEGFR2 (Flk-1) and TUNEL assay. (A) More TUNEL-positive nuclei (red) were observed in tumors treated with soluble EphA-Fc receptor than those treated with control IgG. Total nuclei in tumor sections were visualized by DAPI staining (blue), and endothelial cells were stained by anti–Flk-1 antibodies (green). Endothelial cell apoptosis was shown as double positive for TUNEL and VEGFR2 (yellow in overlaid insert). (B) Total apoptosis in tumor in RIP-Tag/RIP-EphA2-Fc double transgenic mice or in RIP-Tag single transgenic littermates was determined by quantifying the number of pixels in TUNEL-positive nuclei relative to the number of DAPI pixels (total nuclei). The level of endothelial apoptosis and the level of tumor cell apoptosis were determined by the number of pixels of double positive cells (yellow)/the number of DAPI pixels, and the number of pixels of total TUNEL-positive nuclei (red)–double positive cells (yellow)/number of DAPI pixels, respectively. (C) The levels of endothelial and tumor cell apoptosis in RIP-Tag mice treated with soluble EphA receptor or control IgG were calculated as described in (B) (n = 5 independent samples/condition for in vivo analysis). Statistical analysis was performed using the two-tailed, paired Student's t-test. *P < .01; **P < .05 vs control.*

maintain systemic serum concentrations of 50 to 150 ng/ml for the duration of administration.

In the prevention trial, an osmotic pump containing 500 μg of soluble EphA receptor or control IgG protein was implanted into 8-week–old RIP-Tag mice to inhibit the angiogenic switch before the initial formation of solid tumors. Two weeks later (10-week–old mice), angiogenic islets and small tumors were isolated by retrograde infusion of collagenase P and enumerated. Administration of either EphA2-Fc or EphA3-Fc significantly reduced the incidence of angiogenic islet and small tumor formation, compared to animals treated with control IgG (Figure 2*B*). In the intervention trial,



treatment of the RIP-Tag mice began at 10 weeks of age when every mouse developed a few small but highly vascularized solid tumors, and ended 2 weeks later. Consistent with data in RIP-Tag/RIP-EphA2-Fc double transgenic mice, treatment of RIP-Tag mice with soluble EphA-Fc proteins resulted in a two-fold decrease in tumor volume, although there was an increase in small tumor numbers (4.70 ± 1.4 in IgG-treated mice *vs* 8.09 ± 3.5 in EphA3-Fc–treated mice) (Figure 2*C*) compared to mice treated with Fc control, IgG. Soluble EphA receptor treatment also resulted in significantly reduced tyrosine phosphorylation of endogenous EphA2 in tumors, suggesting that these soluble receptors disrupt EphA2 signaling *in vivo* (Figure 2*D*). Taken together, these results suggest that soluble EphA receptors inhibit angiogenic switch and tumor progression *in vivo*.

Treatment with Soluble EphA Receptors Resulted in Increased Apoptosis in Both Tumor Cells and Tumor Endothelium In Vivo

Because our prior studies showed that soluble EphA receptor reduced vessel density in 4T1 mammary tumor grafts [9], we investigated whether similar changes occur in islet cell carcinoma in RIP-Tag mice between mice treated with soluble EphA receptors and those treated with control proteins. Interestingly, soluble EphA2 receptors did not cause significant changes in microvessel density in islet tumor (data not shown). It has been recently recognized that during tumor progression under antiangiogenic therapy, microvessel density can decrease, increase, or remain unchanged, depending on the relative rates of cell death between tumor cells and tumor endothelium in a given tumor [18]. Accordingly, we measured the apoptosis levels of tumor and endothelial cells simultaneously by dual staining of TUNEL-positive nuclei and an endothelial cell–specific marker, Flk-1 (VEGF receptor 2). As shown in Figure 3, both tumor cells and endothelial cells within tumors treated with EphA2-Fc or EphA3-Fc displayed increased levels of TUNEL-positive nuclei relative to those of control IgG-treated tumors. Thus, it is likely that in treated 4T1 mouse mammary carcinomas, capillary dropout exceeds tumor cell death so that vessel density decreases, whereas in treated islet carcinomas, dropout rates of capillaries and tumor cells are similar, resulting in unaltered microvascular density. Because βTCs do not express significant levels of EphA2 receptor and soluble EphA2-Fc do not affect the growth or apoptosis in cultured tumor cells (data not shown), increased tumor cell apoptosis is likely due to diminished blood supply in the treated tumor.

Soluble EphA Receptors Block Tumor Cell–Conditioned Medium- and VEGF-Induced Angiogenic Responses

Based on our previous studies demonstrating that ephrin-A1 ligands and EphA2 receptors are expressed complementarily on tumor cells and vascular endothelial cells [9], we hypothesize that ephrin-A1 expressed on the tumor cells recruits blood vessels through activation of endothelial EphA2 receptors. However, because ephrin ligands are membrane-bound and soluble monomeric ligands are not functional [19–21], it is not clear how tumor cells could stimulate an angiogenic response from vessels that are not in direct contact with the tumor. Bergers et al. [22] reported that islet carcinoma expresses VEGF, and blockade of the VEGF receptor tyrosine kinase by the chemical inhibitor SU5416 inhibits angiogenic islet formation and tumor burden in RIP-Tag mice, suggesting that VEGF is a long-range angiogenic signal produced by the islet tumor cells.

As a first step to dissect the mechanism of soluble EphA receptor–mediated inhibition of tumor angiogenesis, we tested whether soluble EphA receptor can block tumor cell (βTC)–conditioned medium-induced endothelial cell migration. As shown in Figure 4*A*, βTC-conditioned medium induced HUVEC migration, and this response was blocked by either VEGF-neutralizing antibodies or soluble EphA2-Fc, suggesting that soluble EphA2-Fc receptor can block VEGF-induced angiogenic response. Consistent with this observation, βTC-conditioned medium induced the phosphorylation of EphA2 receptor (Figure 4*B*). To further investigate whether soluble EphA receptors administered systemically can inhibit VEGF-induced angiogenesis *in vivo*, hydron pellets impregnated with VEGF were surgically implanted into mouse corneal stromal micropockets, and mice were treated daily for 5 days with either 400 μg of control IgG or soluble EphA3-Fc receptors by intraperitoneal injection. Treatment with soluble EphA3-Fc receptors significantly inhibited VEGF-induced corneal angiogenesis (Figure 4*C*). These data, together with the fact that soluble EphA receptors inhibit VEGF-dependent islet tumor progression, suggest an essential role for EphA receptor function in VEGF-dependent angiogenesis.

EphA2 Receptor Activity Mediates Cellular Interactions between Tumor and Endothelial Cells

To investigate whether it is the EphA2 receptor specifically, rather than other members of the EphA family, that plays a role in βTC-induced endothelial cell migration, we utilized a dominant negative form of EphA2 cytoplasmic

Figure 4. *Soluble EphA receptors inhibit βTC-conditioned medium and VEGF-induced angiogenic responses. (A) Soluble EphA2-Fc inhibits tumor cell–conditioned medium and VEGF-induced endothelial cell migration. Endothelial cell migration was determined by wound closure assay. Replicate circular "wounds" were generated in confluent HUVEC monolayers, and treated with βTC condition medium in the presence of anti-VEGF neutralizing antibodies or soluble EphA2-Fc. Residual fractional "wound" areas were measured following a time course. Medium: DMEM/2% FBS. (B) Tumor cell conditioned medium induces phosphorylation of EphA2 receptor. HUVECs were serum-starved and stimulated with βTC-conditioned media (βTC-CM) or ephrin-A1 at indicated times. Cell lysates were immunoprecipitated with an anti-EphA2 antibody and subjected to Western blot analysis using anti-PY antibodies pY99 and pY20. (C) Systemic administration of soluble EphA3-Fc inhibits VEGF-induced mouse corneal angiogenesis. Hydron/sucralfate pellet impregnated with rhVEGF (160 ng/pellet) was implanted into mouse cornea as described in Materials and Methods section. Mice were treated systemically by daily intraperitoneal injection of 400 μg of EphA3-Fc (solid bar) or Fc control protein, IgG1 (shaded bar), beginning 1 day prior to implantation. The percentage of vascularized area was determined by the number of blood vessel pixel/whole corneal area using Spot software (n = 6 per experimental group). Statistical analysis was performed using the two-tailed, paired Student's t-test.*

domain truncation mutant (EphA2-ΔC) and an gain-of-function EphA2 mutant (EphA2-Neu) that is autophosphorylated in the absence of ligand stimulation (Figure 5*A*). Microvascular endothelial cells were transfected with plasmids expressing either wild-type EphA2, EphA2-Neu, EphA2-ΔC, or control pGABE, and approximately 50% of cells were transfected judging by GFP expression. Western blot analysis shows that mutant EphA2 proteins are overexpressed, compared with endogenous endothelial EphA2 in control cells (Figure 5*B*). The transfected cells were then tested for the ability to migrate toward βTCs in a coculture assay. Endothelial cells expressing EphA2-NeuTM showed a significant increase in migration to βTCs, compared to those transfected with control pGABE. In contrast, expression of EphA2-ΔC resulted in a significant decrease in migration toward βTCs compared to controls (Figure 5*C*). These data

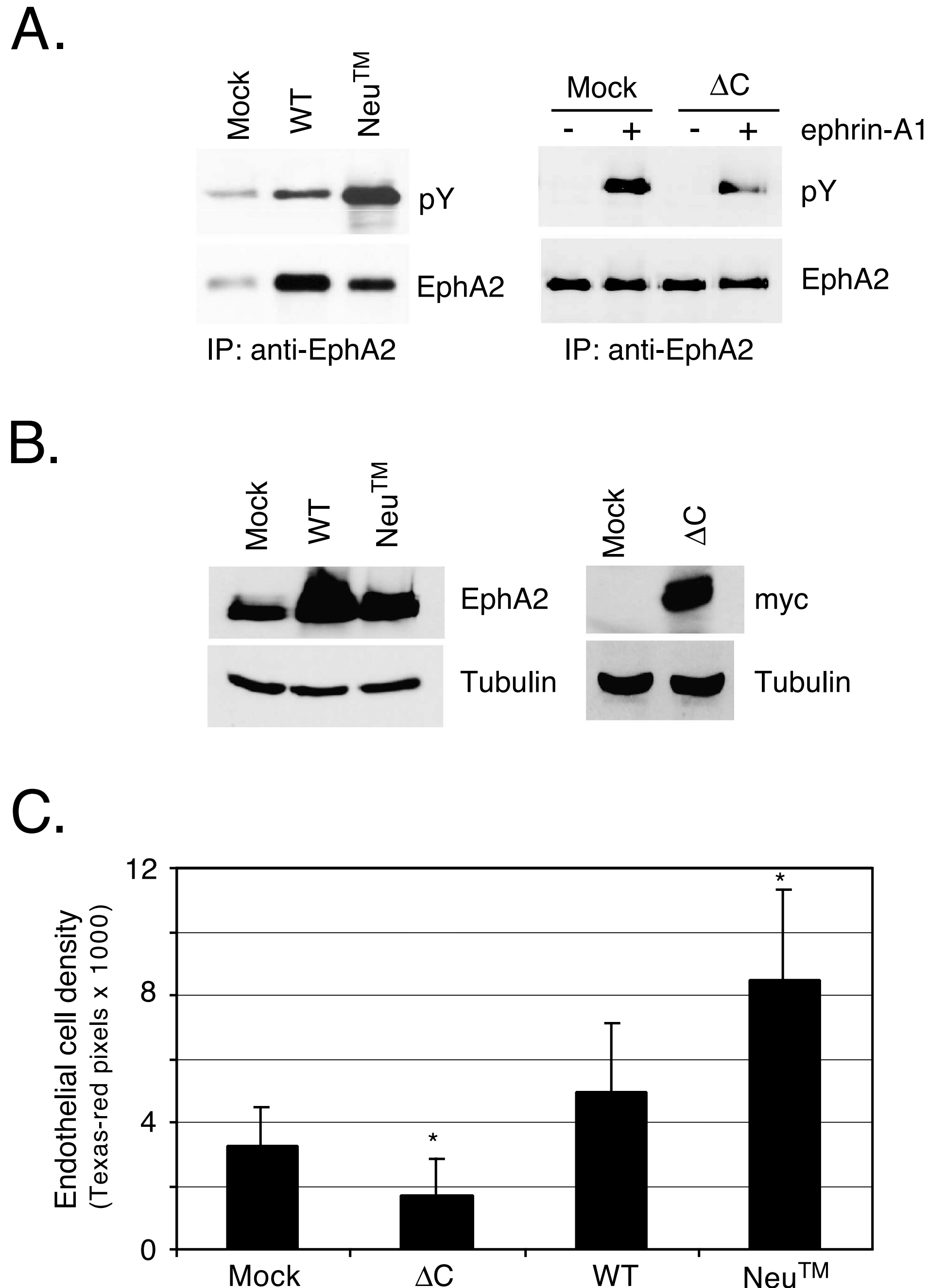


Figure 5. *Inhibition of EphA2 receptor activation blocks tumor cell–induced endothelial cell migration. (A) Expression of EphA2-ΔC inhibits, and EphA2-Neu enhances, phosphorylation of EphA2 receptor. EphA2 was immunoprecipitated from transfected COS cells and probed for PY, and EphA2 levels were assessed by probing for EphA2 expression. (B) Primary BPMECs were transfected with wild-type (WT) EphA2, EphA2-ΔC (ΔC), or EphA2-Neu (Neu), and the expression of wild-type and EphA2 mutant proteins was confirmed by Western blot analysis using antibodies against myc (1:500), EphA2 (1:500), or tubulin (1:1000). (C) Transfected BPMECs were tested for their ability to migrate to βTCs in a tumor cell–endothelial cell coculture assay as described in Materials and Methods section. Statistical analysis was performed using the two-tailed, paired Student's t-test. *P < .01 EphA2-ΔC or EphA2-Neu vs control.*

suggest that the EphA2 receptor mediates the angiogenic response initiated by tumors at the level of endothelial cell migration, providing further support for a proangiogenic role for EphA2 receptors in tumor progression.

Discussion

An elevated expression of Eph receptors has long been correlated with the growth of solid tumors. However, the functional role of this family of receptor tyrosine kinases in carcinogenesis and tumor angiogenesis has just begun to be investigated. In particular, expression of ephrin-A1 ligand and EphA2 receptor has recently been shown in a number of human cancers and tumor vasculature [8]. We previously reported the expression of this pair of ligand–receptor in the vasculature of two types of murine cancers, and that blocking EphA receptor activation inhibits tumor angiogenesis in a vascular window assay and a subcutaneous graft model of 4T1 mammary carcinoma cells [9]. Work presented in this manuscript advanced our previous findings in several respects. First, this report investigated the function of EphA receptor tyrosine kinases in an *in vivo* tumor model (RIP-Tag transgenic mice), where tumors arise in an endogenous environment and tumor development is multistage. Thus, we show that soluble EphA-Fc receptors not only inhibited tumor growth and decreased tumor volume, but also had a negative effect on pretumor lesions, the angiogenic islets. Second, although there are many differences between 4T1 breast cancer and islet cell carcinoma, the fact that soluble EphA receptor inhibited both types of tumor suggests that tumor endothelium is a common target of EphA-Fc proteins and that inhibition of tumor angiogenesis is a general mechanism of suppressing tumor progression by soluble EphA receptors. Third, instead of *in vitro* treatment and local delivery of soluble receptors in our previous studies, inhibition of tumor progression through a systemic and efficient method of delivering soluble EphA proteins through an osmotic pump indicates a possible relevance for EphA targeting in clinical cancer therapeutics. Finally, using a dominant negative form of EphA2 cytoplasmic domain truncation mutant (EphA2-ΔC) and a gain-of-function EphA2 mutant (EphA2-Neu), we provided evidence that tumor cell–induced endothelial cell migration is, at least in part, through EphA2 receptor activation.

What is the mechanism of soluble EphA receptor–mediated inhibition of tumor progression *in vivo*? The use of Fc chimeric fusion protein raises the possibility that the binding of EphA2-Fc or EphA3-Fc to ephrin-A1 on tumor cells may initiate complement and/or Fc receptor–mediated cytotoxicity and clearance of tumor cells. We do not favor this hypothesis. The EphA3-Fc fusion protein, which contains mutations in the Fc region that inhibit interactions with complement and Fc receptor, produces effects comparable to EphA2-Fc on angiogenic islet formation and tumor volume. Furthermore, our data derived from RIP-EphA2-Fc/RIP-Tag double transgenic mice, in which soluble EphA2-Fc is expressed as a part of the host genome, yielded similar results as exogenously delivered EphA2-Fc. These data suggest that the effect is due to blocking of endogenous class A Eph ligand–receptor interactions rather than immune-mediated clearance of ephrin-A1 expressing carcinoma or endothelial cells.

Given recent reports of reverse signaling through ephrin ligands upon stimulation with Eph receptors, it is also possible that soluble EphA-Fc receptors could initiate ephrin-mediated reverse signaling in ephrin-A1 expressing tumor cells and/or endothelial cells. Although we cannot rule out the modulation of ephrin-A signaling by treatment with soluble Eph receptors, soluble EphA receptor stimulation alone does not induce angiogenesis in the mouse cornea, nor does it alter endothelial cell migration, sprouting, proliferation, or apoptosis (data not shown). Moreover, reduced phosphorylation of EphA2 receptor in endothelial cells by overexpression of a EphA2-ΔC mutant (Figure 5) mimics the inhibitory effects of the soluble receptor on endothelial cell migration, favoring the interpretation of disruption of endothelial endogenous EphA class receptor function by soluble receptors. Finally, EphA-Fc treatment blocks tyrosine phosphorylation of endogenous EphA2 receptor relative to control (Figure 2*D*), supporting the inhibitory function of soluble EphA receptors on EphA class signaling.

Our data support the hypothesis that soluble EphA receptors block endogenous class A Eph ligand–receptor interaction, thereby affecting recruitment of blood vessels by tumor cells and inhibiting tumor progression *in vivo*. EphA2-Fc treatment inhibits endogenous EphA2 receptor phosphorylation in islet cell carcinoma relative to control IgG (Figure 2*D*), supporting the inhibitory function of soluble EphA receptors on EphA class signaling. Furthermore, soluble EphA receptors block tumor-conditioned medium-induced endothelial cell migration and VEGF-induced angiogenic responses *in vitro* (Figure 4). Administration of soluble EphA receptors *in vivo* inhibited endothelial cell growth and survival within the intact tumor (Figure 3). Taken together, these data suggest that soluble EphA receptors block endogenous EphA class receptor function on endothelial cells to inhibit recruitment of new blood vessels by tumors, thereby suppressing angiogenic islet formation and tumor progression in multistage carcinogenesis *in vivo*.

Nevertheless, because the soluble EphA receptor is administered *in vivo*, the endothelial cell may not be the only target, and other cell types may also be involved in the inhibition of tumor progression by soluble EphA-Fc receptors. One such possible target is the tumor cell. However, βTC tumor cells do not express significant levels of EphA2 receptor or other class A receptors, as judged by Western blot analysis, immunohistochemistry, or RNAse protection assay [9] (Cheng et al., unpublished results). To distinguish the possibility of a direct effect of tumor cells by soluble EphA-Fc receptors, we also performed proliferation and apoptosis assays on cultured βTCs. Our data revealed that EphA-Fc affects neither cultured βTC tumor cell growth nor survival (data not shown). Another possible target is inflammatory infiltrating cells. Although we have not specifically looked for changes in macrophages, neutrophils, or mast

cells in tumor, we do not observe a significant difference in the total amount of infiltrating cells in H&E–stained tumor sections between EphA-Fc – and IgG-treated samples.

EphA2 receptor signaling is known to be required for TNF-α–induced angiogenesis. Pandey et al. [23] showed that ephrin-A1 is a chemoattractant for endothelial cells and that ephrin-A1–neutralizing antibodies inhibited TNF-α–induced cornea angiogenesis. In addition to a role of ephrin-A1 in TNF-α–induced angiogenesis, here we show that EphA receptor signaling also plays a role in VEGF-dependent tumor angiogenesis. Our *in vitro* studies show that RIP-Tag tumor cells produce soluble factors that act upon endothelial cells to induce cell migration. Endothelial migration was inhibited in the presence of either VEGF-neutralizing antibodies or soluble EphA2 receptor (Figure 4*A*). These results indicate that VEGF is a primary angiogenic factor in the tumor condition medium that induces cell migration, and soluble EphA2-Fc blocks VEGF-induced angiogenic response. The role of class A Eph ligand/receptor function in VEGF-induced angiogenesis is further supported by *in vivo* data that administration of soluble EphA receptor systemically blocks VEGF-induced corneal angiogenesis (Figure 4*B*) and VEGF-dependent RIP-Tag angiogenic islet formation and development of solid islet cell carcinoma (Figures 1 and 2).

In summary, our findings suggest that class A Eph receptor regulates tumor angiogenesis and that EphA signaling pathways may represent an attractive novel target for antiangiogenic therapy in cancer.

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