

# EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation

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## Summary

Angiogenesis is critical for vascular remodeling during development and contributes to the pathogenesis of diseases such as cancer. Targeted disruption of several EphB class receptor tyrosine kinases results in vascular remodeling defects during embryogenesis. The role of EphA class receptors in vascular remodeling, however, is not well-characterized. We recently demonstrated that global inhibition of EphA receptors disrupts endothelial migration induced by ephrin, VEGF or tumor-derived signals, though the specific target remained undefined. Here, we report that EphA2 regulates endothelial cell assembly and migration through phosphoinositide (PI) 3-kinase-mediated activation of Rac1 GTPase in two model systems: primary bovine and murine pulmonary microvascular endothelial cells. EphA2-deficient endothelial cells fail to undergo vascular assembly and

migration in response to ephrin-A1 in vitro. Ephrin-A1 stimulation induces PI3-kinase-dependent activation of Rac1 in wild-type endothelial cells, whereas EphA2-deficient cells fail to activate Rac1 upon stimulation. Expression of dominant negative PI3-kinase or Rac1 inhibits ephrin-A1-induced endothelial cell migration. Consistent with in vitro data, EphA2-deficient mice show a diminished angiogenic response to ephrin-A1 in vivo. Moreover, EphA2-deficient endothelial cells fail to assemble in vivo when transplanted into recipient mice. These data suggest that EphA2 is an essential regulator of post-natal angiogenesis.

Key Words: EphA2, Ephrin-A1, Endothelial cell, Rac1, PI3-kinase, Angiogenesis

## Introduction

Angiogenesis is a complex, multi-stage process by which new blood vessels are formed from pre-existing vasculature. Two critical steps in this process are endothelial cell migration and assembly into new tubules. Over the last decade, a diverse array of molecular regulators that participate in the process of angiogenesis have been identified. The Eph family of receptor tyrosine kinases (RTKs) is one such family of angiogenesis regulators that play a prominent role in endothelial cell assembly and migration.

The Eph family of receptor tyrosine kinases and their membrane-tethered ligands, known as ephrins, constitute the largest RTK sub-family, with at least 14 receptors and nine ligands (Cheng et al., 2002a; Kullander and Klein, 2002). This family is subdivided into class A receptors that bind GPI-tethered A class ephrins, and B class receptors that bind transmembrane-tethered B class ephrins. Gene targeting studies have established several class B Eph family members as key regulators of embryonic vascular development. For example, targeted disruption of ephrin-B2 ligand, which is

expressed in arterial but not venous endothelial cells, results in embryonic lethality because of defects in remodeling of primitive capillary beds into large and small vessels (Wang et al., 1998). Mice lacking EphB4 receptor, a marker for embryonic venous endothelial cells, phenocopy ephrin-B2 deficiency, suggesting that these two molecules interact in the regulation of angiogenesis during development (Gerety et al., 1999). Combined deficiency of EphB2 and EphB3 receptors also results in death from angiogenic defects in approximately 30% of mutant embryos (Adams et al., 1999). These data support the crucial role for EphB-mediated regulation of developmental angiogenesis.

Class A Eph receptors have been shown to regulate post-natal angiogenesis in adults. Earlier studies showed that ephrin-A1 stimulates endothelial cell migration in culture and induces corneal angiogenesis in vivo (Pandey et al., 1994), suggesting that activation of A class Eph RTK could regulate vascular remodeling in mature tissues. The role of class A Eph receptors in regulating endothelial cell migration and assembly is further supported by studies in which ephrin-A1 ligand was

shown to promote endothelial cell assembly into capillary-like structures (Daniel et al., 1996). Loss of EphA2 function by overexpression of a dominant negative receptor mutation inhibited endothelial cell assembly in vitro, suggesting that EphA2 RTK facilitates angiogenic remodeling (Ogawa et al., 2000). More recently, we reported that blockade of class A Eph receptor activation through use of soluble EphA2-Fc or EphA3-Fc receptors inhibited endothelial cell migration and sprouting in response to ephrin-A1 or in response to tumor cells in co-culture assays (Brantley et al., 2002; Cheng et al., 2002b). Moreover, these soluble receptors also inhibited tumor angiogenesis and progression in vivo (Brantley et al., 2002; Cheng et al., 2003). However, since ephrin-A1 can bind and activate multiple EphA receptors, and since soluble Eph receptors are capable of disrupting signaling through multiple endogenous A class Eph RTK, it is unclear which A class receptor(s) is responsible for downstream signaling linked to angiogenic responses, particularly in adult animals. In addition, despite identification of multiple signaling molecules downstream of EphA class receptors, signal transduction pathways that link EphA receptor activation to cell motility in endothelial cells have not been delineated.

In this report, we demonstrate that EphA2 RTK regulates vascular assembly and migration of primary microvascular endothelial cells. Endothelial cells isolated from EphA2-deficient mice fail to assemble into capillary-like structures and display defective ephrin-A1-induced migration in vitro and in vivo. Defects in migration and assembly were rescued by restoring EphA2 expression, demonstrating that EphA2 RTK is required in endothelial cell migration. In addition, EphA2-deficient mice display decreased angiogenesis in response to ephrin-A1 in vivo. Ephrin-A1 stimulation induces PI3-kinase-dependent activation of Rac1 in wild-type endothelial cells, whereas EphA2-deficient cells fail to activate Rac1 upon stimulation. Expression of dominant negative PI3-kinase or Rac1 inhibits ephrin-A1-induced endothelial cell migration. Thus, PI3-kinase-dependent Rac1 activity is essential in ephrin-A1-mediated endothelial cell migration. Taken together, these data suggest that EphA2 is a key regulator of angiogenic remodeling in adult endothelium.

## Materials and Methods

### Plasmids, adenoviruses and reagents

The gain-of-function EphA2 receptor mutant (EphA2-Neu<sup>TM</sup>) was generated by substituting the transmembrane domain of EphA2 with that from oncogenic neu/erbB2, which was generously provided by Dr Alastair Reith (Ludwig Institute, London). The myc-tagged dominant negative EphA2 cytoplasmic domain truncation mutant (EphA2-ΔC) was generated by PCR amplification of the extracellular and transmembrane domain of EphA2 and subsequent subcloning into pcDNA 3.1 (Invitrogen). Expression constructs encoding dominant negative myc-Rac1-N17 and Δ-p85 were described previously (Anastasiadis et al., 2000; Eder et al., 1998). Recombinant adenovirus encoding wild-type EphA2 was generated using the Ad-Easy system according to the manufacturer's protocol (Qbiogene Incorporated) (He et al., 1998). Recombinant adenovirus encoding β-galactosidase was described previously (Cheng and Chen, 2001). Expression of recombinant constructs and adenoviruses was confirmed by western blot analysis. Antibodies used for immunoblot include: anti-myc (1:500; Cell Signaling Technology), anti-EphA2 (1:500) and phosphotyrosine antibodies (1:250; Santa Cruz Biotechnology), anti-β-galactosidase (Chemicon, 1:500), anti-tubulin (1:1000; Sigma-

Aldrich), anti-Rac1 and anti-cdc42 antibodies (1:250; Pharmingen), anti-phospho-p38 and anti-p38 MAPK antibodies (1:500; Cell Signaling Technology), anti-p85 (1:1000; Upstate Biotechnology). A mixture of polyclonal anti-EphA2 (0.5 μg; C-20, Santa Cruz Biotechnology) and monoclonal anti-EphA2 (1 μg; D7, Upstate Biotechnology) antibodies were used to immunoprecipitate EphA2 from endothelial cell lysates. Two micrograms of anti-p85 (Upstate Biotechnology) antibody was used to immunoprecipitate p85 from endothelial cell lysates. For immunohistochemical detection of CD31, 20 μg/ml anti-CD31 antibody (Pharmingen) was used as described previously (Brantley et al., 2002). For immunohistochemical detection of Ki67 expression, a 1:500 dilution of anti-Ki67 antibody (Novocastra Laboratories) was used.

### Disruption of the *EphA2* gene via homologous recombination

The mouse *EphA2* genomic locus was isolated from a 129 strain genomic phage library. An *EphA2* replacement vector (pT1.ephA2) contained bacterial *neo* and HSV-TK gene was used to disrupt the *EphA2* gene within the extracellular domain [at a unique *Hind*III site at nucleotide 1372 corresponding to amino acid residue 426; accession number NM\_010139 (Ruiz and Robertson, 1994)]. The *Hind*III site is located within an exon containing nucleotides 1076-1395 of the *EphA2* cDNA. For simplicity, only the exon containing the *Hind*III site is shown in Fig. 1. The targeted gene is expected to encode a nonfunctional protein truncated at amino acid residue 426 (within the extracellular domain). The original mutant mice were maintained on an 129×C57BL/6 mixed background, and were subsequently backcrossed into the BALB/c genetic background for one generation to produce wild-type, heterozygous and null animals. The wild-type and targeted alleles were distinguished by Southern blot analysis.

### Endothelial cells

Bovine pulmonary microvascular endothelial cells (BPMEC; VEC Technologies) were maintained in EGM medium (Clonetics) with 10% fetal bovine serum (FBS; Hyclone) or in DMEM (Mediatech) with 1% FBS starvation medium where indicated. Murine pulmonary microvascular endothelial cells (MPMEC) were isolated from 1- to 3-month-old EphA2-deficient mice, or wild-type and heterozygous littermates, as described previously (Pozzi et al., 2000) and maintained in EGM-2 medium (Clonetics). Endothelial cell purity was greater than 95% in these cultures, as determined by expression of CD31 endothelial cell marker (data not shown). For transfection experiments, BPMEC were seeded into six-well plates and transfected with 1 μg plasmid DNA using Lipofectamine-2000 (Invitrogen) according to manufacturer's instructions. For adenovirus-mediated expression of EphA2 or β-galactosidase (βgal), BPMEC were seeded into 10 cm dishes and transduced with 10<sup>9</sup> pfu/ml virus for 48 hours prior to migration assays.

### In vitro vascular assembly assay

In vitro vascular assembly assays were performed as described previously (Daniel et al., 1996). Briefly, 12-well plates were coated with 100 μl of growth factor-reduced Matrigel (Becton-Dickinson). After 24 hours starvation in 1% FBS, 24,000 MPMEC were plated in wells in the presence or absence of ephrin-A1 (1 μg/ml; R&D Systems) and photodocumented after 9 hours. Images were acquired on an Olympus CK40 inverted microscope through an Optronics DEI-750C CCD Video Camera using Scion Image 1.62c capture software. Degree of assembly was quantified by counting the number of intersections between branches in assembled endothelial cell networks per 10× field in four random fields from each well, with triplicate samples per condition. For assembly rescue assays, MPMEC were transduced with 10<sup>9</sup> pfu/ml EphA2 or βgal viruses for 48 hours prior to assembly assays.

### Migration assays

Migration assays using 6.5 mm, 8  $\mu$ m pore size Transwells (Costar) were performed as described previously (Brantley et al., 2002). Both sides of the transwell were coated with a 1/20 dilution of growth factor-reduced Matrigel, and blocked with 1% bovine serum albumin. After 24 hours starvation in 1% FBS, cells (100,000/well) were seeded into the upper chamber of transwells in the presence or absence of ephrin-A1 (5  $\mu$ g/ml) in the lower chamber. After 5 hours, cells on the lower surface were counted in three random fields from each well, with triplicate samples per condition. No differences in migration were observed in response to dimeric ephrin-A1-Fc versus ephrin-A1-Fc pre-clustered using anti-human IgG (R&D systems), neither were there any differences in phosphorylation of EphA2 in response to dimeric versus clustered ephrin-A1-Fc (data not shown). For toxin B studies, BPMEC were pre-treated with 100 ng/ml *Clostridium difficile* toxin B (Calbiochem) for 1.5 hours prior to migration assay (Connolly et al., 2002). For LY294002 studies, BPMEC were pre-treated with 50  $\mu$ M LY294002 (Calbiochem) or dimethyl sulfoxide (DMSO; Sigma) vehicle control for 1 hour prior to the migration assay.

### Immunoprecipitation and immunoblot analysis

EphA2 immunoprecipitation was described previously (Brantley et al., 2002). For Rac1 and cdc42 activation assays, BPMEC or MPMEC were serum-starved for 24 hours in 1% FBS followed by stimulation with ephrin-A1 (1  $\mu$ g/ml). Lysates were prepared and incubated with Pak-1 binding domain (PBD)-GST beads (Upstate Biotechnology) as described in the manufacturer's protocol to pull-down GTP-bound Rac1 and/or cdc42. Activated Rac1 and cdc42 (or total Rac1 and cdc42 in lysates) was detected by immunoblot using anti-Rac1 or anti-cdc42 antibodies. For PI3K immunoprecipitation, mock transfected or  $\Delta$ -p85-transfected cell lysates were incubated with 2  $\mu$ g of anti-p85 antibody for 1 hour followed by incubation with A/G Sepharose beads (Santa Cruz Biotechnology) for 1 hour at 4°C. The beads were washed in lysis buffer four times, then fractionated and probed for endogenous p85 or truncated  $\Delta$ -p85 proteins (Eder et al., 1998). For PI3K inhibition studies, cells were pre-incubated with 100 nM wortmannin, 50  $\mu$ M LY294002, or DMSO vehicle control for 10 minutes prior to stimulation/PBD-GST pull-down as described previously for endothelial cells (Zeng et al., 2002). Relative levels of GTP-bound Rac1 and cdc42 were quantified by densitometry using Scion Image 1.62c software analysis.

### In vivo angiogenesis and vascular assembly assays

Sponge assays for angiogenesis were performed as described previously (McCarty et al., 2002). Briefly, gelfoam sponges (Pharmacia & Upjohn) were cut into small pieces (2.5-3 mm wide  $\times$  5 mm long) and soaked with 100  $\mu$ l of PBS containing 10  $\mu$ g ephrin-A1 or IgG. The sponges were then implanted into the subcutaneous dorsal flank of recipient mice. Each recipient received one ephrin-A1-treated sponge and one control IgG sponge implanted in the opposite flank. After 7 days, the mice were injected with a 2% TRITC-dextran ( $M_r$  65)/PBS solution to label host blood vessels (Brantley et al., 2002), and the sponges were collected and analyzed. Whole-mount images were acquired on an Olympus CK40 inverted microscope through an Optronics DEI-750C CCD Video Camera using Scion Image 1.62c capture software. The sponges were then paraffin-embedded, and 7  $\mu$ m sections prepared, rehydrated, and counterstained with DAPI (Sigma). Fluorescent images were acquired on an Olympus BX60 microscope through an Optronics DEI-750C CCD Video Camera using Scion Image 1.62c capture software. The TRITC and DAPI fields were merged using Adobe Photoshop 5.5 software.

In vivo vascular assembly assays were performed using MPMEC isolated from *EphA2*<sup>+/+</sup> or *EphA2*<sup>-/-</sup> donor mice suspended in Matrigel 'plugs' (Brantley et al., 2002). As above, endothelial cell purity was

judged to be greater than 95% in these cultures based on expression of CD31 endothelial cell marker (data not shown). Prior to transplantation, the cells were transduced with 10<sup>9</sup> pfu/ml  $\beta$ gal virus, 1 dose/day for 2 days. MPMEC (1 $\times$ 10<sup>6</sup> cells) were suspended in 300  $\mu$ l growth factor-reduced Matrigel and the mixture was injected subcutaneously into the dorsal flank of 6-week-old athymic nude mice (Harlan Sprague Dawley). Plugs were collected after 4, 7 and 10 days and 10  $\mu$ m cryosections were prepared and examined for  $\beta$ -galactosidase activity by X-gal staining as described previously (Chen et al., 1996). The sections were counterstained with Eosin, mounted and photodocumented. Images were acquired on an Olympus BX60 microscope through an Optronics DEI-750C CCD Video Camera using Scion Image 1.62c capture software. For immunohistochemical analysis, sections were stained first with X-gal and then processed for CD31 immunohistochemistry as described previously (Brantley et al., 2002). CD31 immunoreaction was detected by biotinylated secondary antibody (PharMingen) and avidin-peroxidase (Vector Laboratories) reaction with 3,3'-diaminobenzidine tetrahydrochloride substrate (Zymed Laboratories). The sections were lightly counterstained with Eosin, mounted and photodocumented as described above.

### Proliferation and apoptosis assays

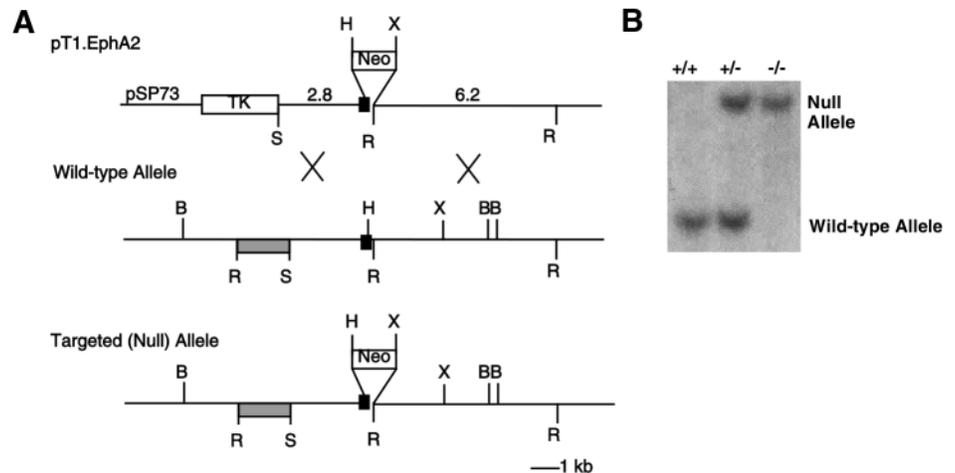
For quantification of proliferation and apoptosis, 10  $\mu$ m cryosections from plugs collected 4 and 10 days post-transplantation were stained with X-gal as described above and processed for Ki67 immunohistochemistry or TUNEL analysis. Ki67 immunoreaction was detected by biotinylated secondary antibody (PharMingen) and avidin-peroxidase (Vector Laboratories) reaction with 3,3'-diaminobenzidine tetrahydrochloride substrate (Zymed Laboratories). TUNEL analysis was performed using an Apoptag red in situ apoptosis detection kit (Serologicals Corporation) as described previously (Brantley et al., 2002). The percentage of *lacZ*<sup>+</sup>/Ki67<sup>+</sup> or *lacZ*<sup>+</sup>/TUNEL<sup>+</sup> nuclei relative to total *lacZ*<sup>+</sup> nuclei in four random 40 $\times$  fields/sample was calculated. Data are representative of three independent samples/condition.

## Results

### EphA2-deficiency impairs vascular assembly in response to ephrin-A1

To determine if EphA2 RTK is required for angiogenesis, EphA2-deficient mice were generated by targeted disruption of the *EphA2* gene through homologous recombination (Fig. 1A). Southern analysis of genomic DNA confirmed the presence of the targeted allele in heterozygous and null animals (Fig. 1B). Consistent with previous studies in which EphA2 was disrupted by gene trap mutagenesis (Chen et al., 1996), EphA2-deficient animals were viable, fertile and displayed no overt anomalies during embryonic development. Since EphA2 is not expressed in the embryonic vasculature (Ruiz and Robertson, 1994), these results suggest that EphA2 is not required for angiogenesis during development.

Expression of EphA2 has been detected in endothelium from mature animals, suggesting that this receptor might regulate post-natal angiogenesis (Brantley et al., 2002; Cheng et al., 2002b; Daniel et al., 1996; Ogawa et al., 2000). To address this hypothesis, we isolated murine pulmonary microvascular endothelial cells (MPMEC) from adult EphA2-deficient mice, as well as wild-type or heterozygous control littermates. Endothelial cells isolated from EphA2-deficient mice expressed no detectable EphA2 protein (Fig. 2E), whereas abundant expression was observed in wild-type and heterozygous MPMEC (Fig. 3A). Consistent with previous



**Fig. 1.** Targeted disruption of the mouse *ephA2* gene. (A) Map of *ephA2* targeting vector, *ephA2* and recombined loci. PT1.*ephA2* consists of a 2.8 kb *EcoRI* fragment (3' homology) flanking a pMCIneo expression cassette. The black box represents *ephA2* exonic sequences that are disrupted by the neo cassette. The gray box between the *EcoRI* and *SacI* sites in the genomic locus map represents a unique sequence located outside the targeting vector sequences used to detect homologous recombination events by Southern blot. B, *BglIII*; H, *HindIII*;

R, *EcoRI*; X, *XbaI*. (B) Southern blot analysis of tail DNA confirmed the presence of the wild-type allele in wild-type (+/+) and heterozygous (+/-) mice and the presence of the targeted allele in heterozygous (+/-) and null (-/-) mice.

data, endothelial cell proliferation and apoptosis/survival in culture was not affected in the absence of EphA2 receptor [data not shown (Cheng et al., 2002a)]. In contrast, endothelial cell assembly into capillary-like structures (vascular assembly) in response to ephrin-A1 was significantly impaired in EphA2-deficient MPMEC relative to the wild-type and heterozygous cells (Fig. 2A,B). Overexpression of wild-type EphA2 in EphA2-deficient cells via adenoviral transduction (Fig. 2E) rescued assembly, even in the absence of ephrin-A1 stimulation (Fig. 2C,D), whereas control  $\beta$ gal virus had no effect on assembly. Transduction efficiency approached 100%, as verified by co-expression of green fluorescent protein (GFP) from an independent CMV-driven GFP cassette within the adenoviral plasmid (Fig. 2C). These data indicate that EphA2 RTK is necessary for ephrin-A1-mediated vascular assembly.

#### Loss of EphA2 receptor expression or modulation of EphA2 kinase activity affects ephrin-A1-induced endothelial cell migration

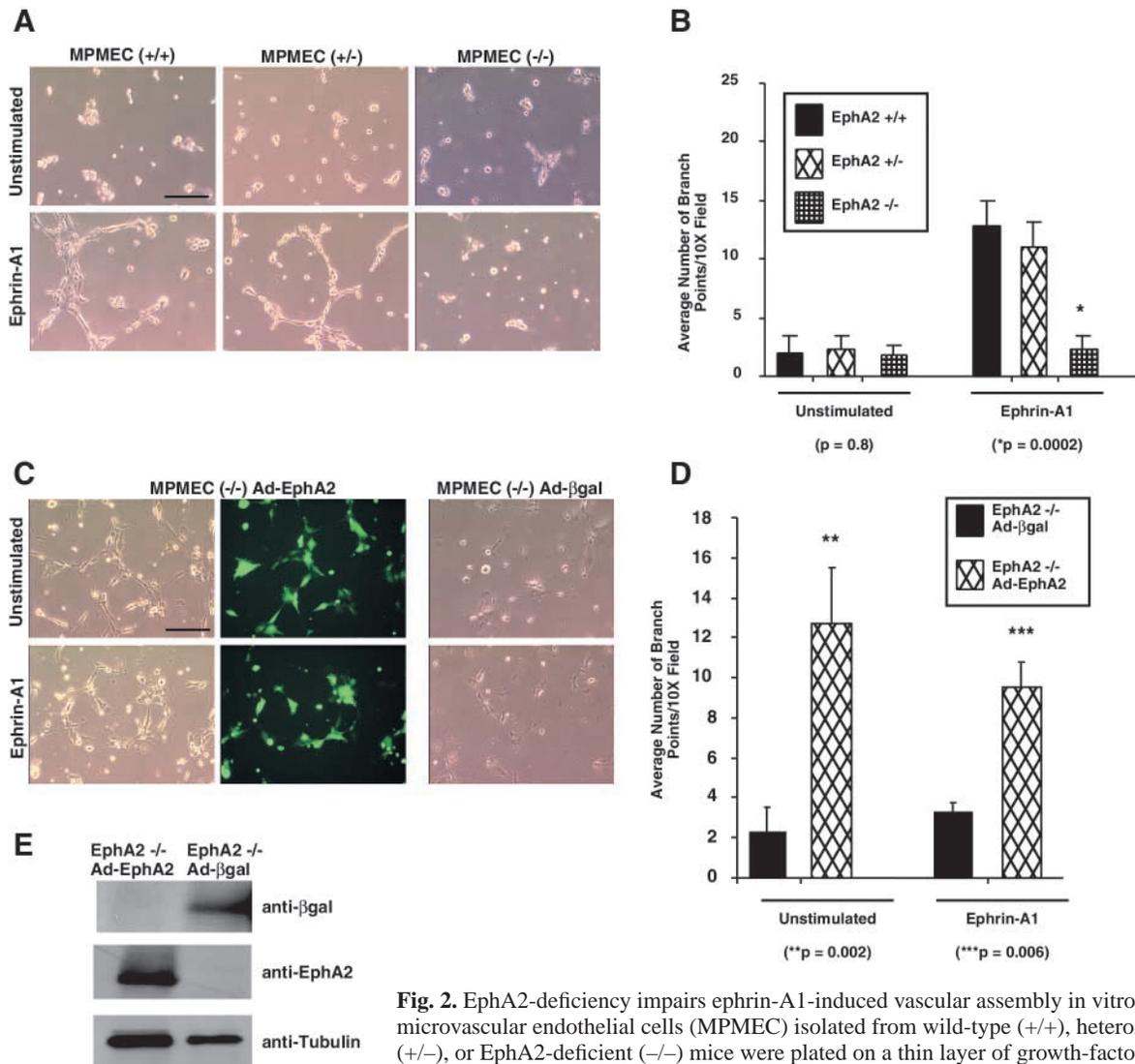
As vascular assembly depends upon endothelial cell migration, we also assessed migration of MPMEC by transwell assay. While ephrin-A1 stimulated migration of endothelial cells derived from wild-type and heterozygous mice, migration of EphA2-deficient MPMEC was significantly impaired (Fig. 3B). No differences in endothelial cell migration or phosphorylation of EphA2 receptor in response to dimeric versus clustered ephrin-A1-Fc were observed (data not shown). Migration defects in EphA2-deficient cells were rescued by adenovirus-mediated expression of wild-type EphA2, but not control  $\beta$ -galactosidase protein (Fig. 3A,B). These data indicate that EphA2 RTK is required for ephrin-A1-mediated vascular assembly and migration.

To further determine whether EphA2 receptor phosphorylation and signaling are critical for endothelial cell migration, we expressed mutant EphA2 receptors that enhanced or diminished EphA2 kinase activity in BPMEC and assessed the effect of these mutants on ephrin-A1-induced migration. Expression of an EphA2 receptor gain-of-function mutant with elevated kinase activity (EphA2-Neu<sup>TM</sup>) enhanced basal and ephrin-A1-induced EphA2 autophosphorylation (Fig. 3C), while

expression of a truncated, dominant negative EphA2 mutant lacking the intracellular cytoplasmic domain (EphA2- $\Delta$ C) significantly impaired endogenous EphA2 autophosphorylation in response to ephrin-A1 (Fig. 3D). Consistent with data from MPMEC, we observed a similar inhibition of ephrin-A1-induced migration in BPMEC expressing EphA2- $\Delta$ C (Fig. 3E). Conversely, expression of EphA2-Neu<sup>TM</sup> enhanced basal level BPMEC migration (Fig. 3E). These data demonstrate that EphA2 receptor activation and signaling regulate endothelial cell migration in response to ephrin-A1 stimulation.

#### Ephrin-A1/EphA2-induced endothelial cell migration is mediated by activation of Rac1 GTPase

We next investigated the molecular mechanisms through which EphA2 regulates endothelial cell migration. Dynamic regulation of the actin cytoskeleton is critical in cell migration, and Rho family GTPases are known to be key regulators of this process and have been shown to be necessary for endothelial cell migration (Connolly et al., 2002; Ridley, 2001; van Nieuw Amerongen and van Hinsbergh, 2001). To identify the specific pro-migratory GTPase(s) activated by ephrin-A1, we stimulated heterozygous or EphA2-deficient MPMEC with ephrin-A1 and assessed activation of Rac1 and cdc42. Cells were stimulated with ephrin-A1, and activated GTP-bound Rac and cdc42 proteins were isolated from lysates by precipitation with Pak-1 p21-binding domain (PBD)-GST fusion proteins. Ephrin-A1 induced activation of Rac1 and cdc42 in heterozygous MPMEC within 2.5 minutes (Fig. 4A). Activation of cdc42 was unaffected by loss of EphA2 expression, suggesting other EphA receptor(s) may regulate cdc42 activity in response to ephrin-A1. In contrast, Rac1 activation was blocked in EphA2-deficient MPMEC, demonstrating that Rac1 is specifically activated downstream of the EphA2 receptor upon stimulation with ephrin-A1. Activation of Rac1 by ephrin-A1 was also observed in BPMEC, with maximal activation between 1 and 5 minutes post-stimulation that was consistent with EphA2 phosphorylation induced by ephrin-A1 (Fig. 4B). To determine the functional relevance of Rac1 in EphA2-mediated endothelial cell migration, we first examined ephrin-



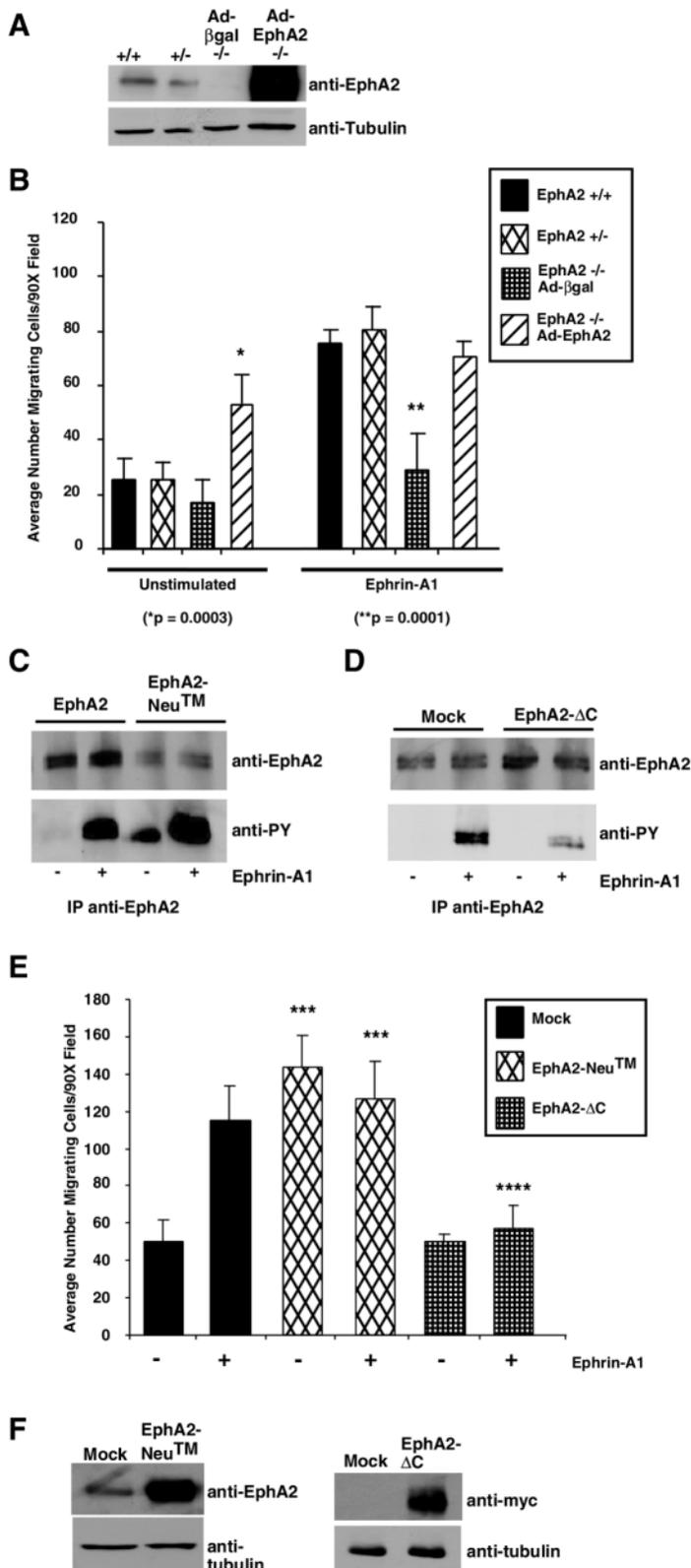
**Fig. 2.** EphA2-deficiency impairs ephrin-A1-induced vascular assembly in vitro. (A) Lung microvascular endothelial cells (MPMEC) isolated from wild-type (+/+), heterozygous (+/-), or EphA2-deficient (-/-) mice were plated on a thin layer of growth-factor-reduced Matrigel in the presence or absence of ephrin-A1 for vascular assembly assay and photographed after 9 hours. Scale bar, 20  $\mu$ m. (B) The numbers of intersections between endothelial cell branches were counted. Four fields per culture were scored for each condition and data are means  $\pm$  s.d. of three independent experiments. Significant differences in assembly for *EphA2*<sup>-/-</sup> cells stimulated with ephrin-A1 (\*) compared to other experimental conditions are indicated for  $P < 0.01$  using ANOVA analysis. (C) EphA2-deficient (-/-) MPMEC were transduced with recombinant adenoviruses encoding wild-type EphA2 (Ad-EphA2) or control  $\beta$ -galactosidase (Ad- $\beta$ gal). After 48 hours, the cells were plated on a thin layer of growth factor-reduced Matrigel in the presence or absence of ephrin-A1 for vascular assembly assay and photographed after 9 hours. Scale bar, 20  $\mu$ m. For MPMEC (-/-) Ad-EphA2, the left hand panels show bright-field images, and the right hand panels show fluorescence images of identical fields displaying co-expression of GFP from the adenovirus plasmid. (D) The numbers of intersections between endothelial cell branches were counted. Four fields per culture were scored for each condition and data are presented as means  $\pm$  s.d. of three independent experiments. Significant differences in assembly are indicated where  $P < 0.01$  (using Student's *t*-test) for *EphA2*<sup>-/-</sup> Ad-EphA2 (\*\*) versus *EphA2*<sup>-/-</sup> Ad- $\beta$ gal and for *EphA2*<sup>-/-</sup> Ad-EphA2 + ephrin-A1 (\*\*\*) versus *EphA2*<sup>-/-</sup> Ad- $\beta$ gal + ephrin-A1. (E) Immunoblot analysis of  $\beta$ gal or EphA2 expression in lysates from MPMEC transduced with recombinant adenoviruses.

assembly. After 9 hours, the endothelial cells were photographed. Scale bar, 20  $\mu$ m. (B) The numbers of intersections between endothelial cell branches were counted. Four fields per culture were scored for each condition and data are means  $\pm$  s.d. of three independent experiments. Significant differences in assembly for *EphA2*<sup>-/-</sup> cells stimulated with ephrin-A1 (\*) compared to other experimental conditions are indicated for  $P < 0.01$  using ANOVA analysis. (C) EphA2-deficient (-/-) MPMEC were transduced with recombinant adenoviruses encoding wild-type EphA2 (Ad-EphA2) or control  $\beta$ -galactosidase (Ad- $\beta$ gal). After 48 hours, the cells were plated on a thin layer of growth factor-reduced Matrigel in the presence or absence of ephrin-A1 for vascular assembly assay and photographed after 9 hours. Scale bar, 20  $\mu$ m. For MPMEC (-/-) Ad-EphA2, the left hand panels show bright-field images, and the right hand panels show fluorescence images of identical fields displaying co-expression of GFP from the adenovirus plasmid. (D) The numbers of intersections between endothelial cell branches were counted. Four fields per culture were scored for each condition and data are presented as means  $\pm$  s.d. of three independent experiments. Significant differences in assembly are indicated where  $P < 0.01$  (using Student's *t*-test) for *EphA2*<sup>-/-</sup> Ad-EphA2 (\*\*) versus *EphA2*<sup>-/-</sup> Ad- $\beta$ gal and for *EphA2*<sup>-/-</sup> Ad-EphA2 + ephrin-A1 (\*\*\*) versus *EphA2*<sup>-/-</sup> Ad- $\beta$ gal + ephrin-A1. (E) Immunoblot analysis of  $\beta$ gal or EphA2 expression in lysates from MPMEC transduced with recombinant adenoviruses.

A1-induced migration of BPMEC in the presence or absence of *C. difficile* toxin B, an inhibitor of Rho family small GTPases. Treatment with toxin B significantly inhibited ephrin-A1-induced endothelial cell migration in mock-transfected BPMEC or in BPMEC expressing the gain-of-function mutant EphA2-Neu<sup>TM</sup> (Fig. 4C). In addition, expression of a dominant negative mutant of Rac1 [Rac1-N17 (Anastasiadis et al., 2000)] markedly impaired BPMEC migration in response to ephrin-A1 (Fig. 4D), suggesting that ephrin-A1- and EphA2-mediated migration requires Rac1

GTPase. Activation of the Rac1 pathway by ephrin-A1 appears to be quite specific, as we did not observe activation of RhoA, JNK or p38 MAPK, and inhibition of p38 MAPK did not block endothelial cell migration in response to ephrin-A1 (data not shown).

PI3-kinase regulates ephrin-A1 induced Rac1 activation and endothelial cell migration  
Phosphoinositide 3-kinase (PI3K) is a well-documented



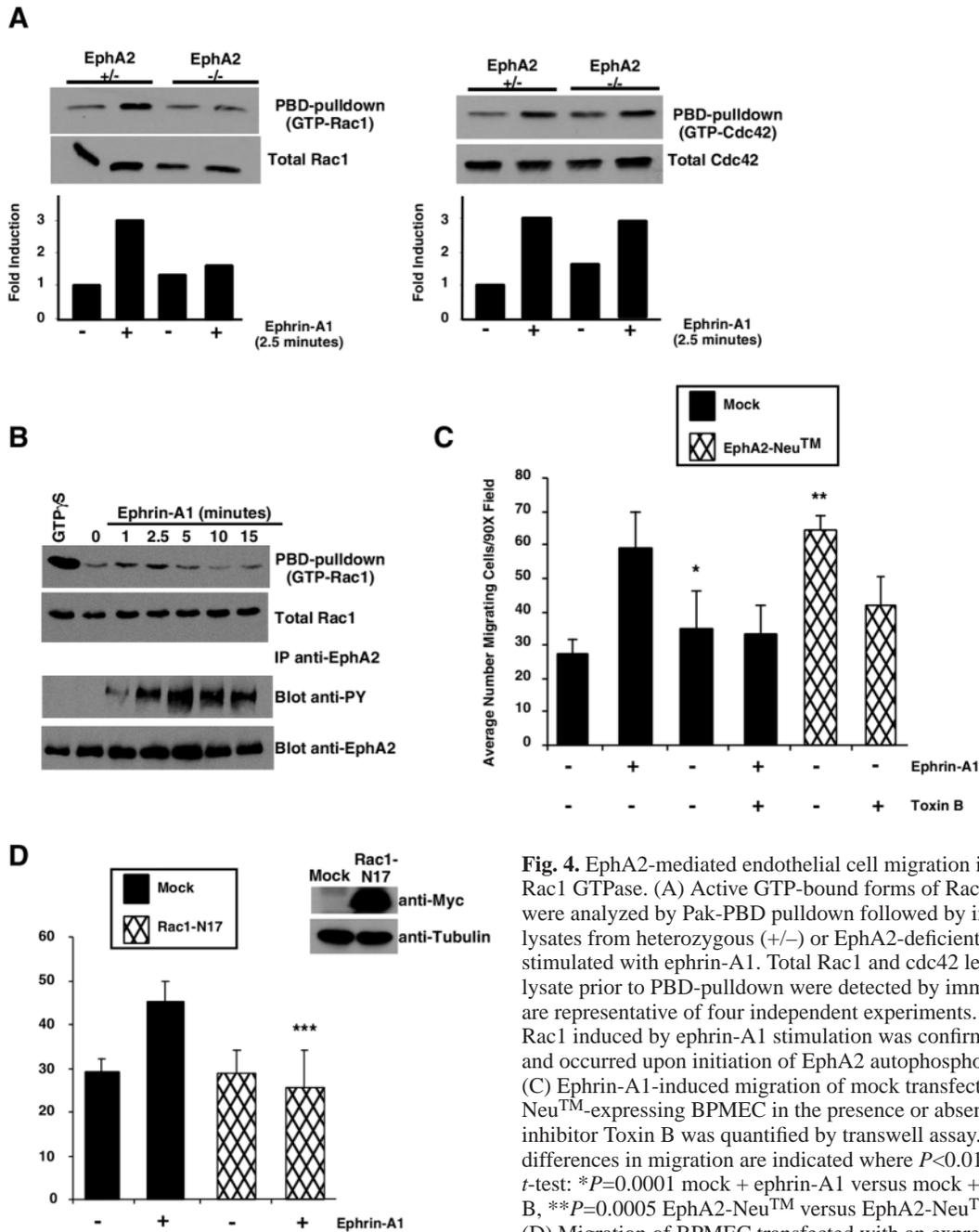
**Fig. 3.** EphA2 receptor is required for ephrin-A1-induced endothelial cell migration. (A) Immunoblot analysis of EphA2 expression in wild-type (+/+), heterozygous (+/-) and Ad-EphA2 transduced EphA2<sup>-/-</sup> MPMEC lysates versus Ad-βgal transduced EphA2<sup>-/-</sup> MPMEC lysates. (B) Migration of MPMEC derived from wild-type, heterozygous, or EphA2-deficient mice in response to ephrin-A1 was quantified by transwell assay. EphA2-deficient MPMECs were infected with recombinant adenoviruses encoding β-galactosidase (EphA2<sup>-/-</sup> Ad-βgal) or wild-type EphA2 (EphA2<sup>-/-</sup> Ad-EphA2) 48 hours prior to migration assay. The number of endothelial cells that had migrated to the lower surface of the transwell were counted. Three fields per transwell were scored for each condition in triplicate samples and data are means±s.d. of three independent experiments. Significant differences in migration for EphA2<sup>-/-</sup> Ad-EphA2 (\*) or Ad-βgal (\*\*) compared to other experimental conditions are indicated where  $P < 0.01$  using ANOVA analysis. (C) Immunoblot analysis of EphA2 immunoprecipitated from BPMEC lysates showing elevated tyrosine phosphorylation of EphA2-Neu<sup>TM</sup> mutant in the absence of ephrin-A1 stimulation. (D) Immunoblot analysis of immunoprecipitated EphA2 showing decreased tyrosine phosphorylation of endogenous EphA2 after ephrin-A1 stimulation in EphA2-ΔC-expressing BPMEC relative to mock transfected cells. (E) Migration of BPMEC expressing kinase elevated EphA2 (EphA2-Neu<sup>TM</sup>) or truncated, dominant negative EphA2 (EphA2-ΔC) in response to ephrin-A1 was also quantified by transwell assay. Significant differences in migration are indicated for  $P < 0.01$  using Student's *t*-test: \*\*\* $P = 0.0001$  EphA2-Neu<sup>TM</sup> +/- ephrin-A1 versus mock unstimulated, \*\*\*\* $P = 0.0005$  EphA2-ΔC + ephrin-A1 versus mock + ephrin-A1. (F) Expression of EphA2-Neu<sup>TM</sup> or EphA2-ΔC in BPMEC was confirmed by immunoblot analysis.

of pharmacologic inhibitors of PI3K, wortmannin or LY294002, and examined activation of Rac1. Ephrin-A1-induced activation of Rac1 was significantly impaired in the presence of either wortmannin or LY294002 (Fig. 5A). Moreover, LY294002 treatment significantly inhibited BPMEC migration in response to ephrin-A1 (Fig. 5B). As an independent approach, we examined activation of Rac1 in BPMEC expressing a dominant negative form of the p85 regulatory subunit of PI3K [Δ-p85 (Eder et al., 1998)]. Expression of Δ-p85 significantly inhibited ephrin-A1-induced activation of Rac1 (Fig. 5C), suggesting PI3K is necessary for ephrin-A1 induction of Rac1. Furthermore, BPMEC expressing Δ-p85 displayed a significant reduction in migration relative to mock-transfected cells (Fig. 5D), suggesting that PI3K is required for Rac1 activation and endothelial cell migration.

#### EphA2-deficient mice display impaired ephrin-A1-induced angiogenesis in vivo

Based on the defects in assembly and migration of EphA2-deficient cells *ex vivo*, we wished to determine if angiogenesis is impaired in EphA2-deficient mice. To test this, we implanted surgical sponges impregnated with control IgG or ephrin-A1 subcutaneously into the dorsal flank of control heterozygous mice and EphA2-deficient littermates. After 7 days, we injected the mice intravenously with a TRITC-dextran solution to visualize host blood vessels associated with sponges. In heterozygous mice, we observed a marked increase in surface vessels associated with ephrin-A1-

upstream regulator of Rac1 activation (Cantrell, 2001), and the p85 regulatory subunit of PI 3-KINASE is known to associate with activated EphA2 RTK (Pandey et al., 1994). To determine if EphA2-mediated activation of Rac1 is PI3K-dependent, we stimulated BPMEC with ephrin-A1 in the presence or absence



**Fig. 4.** EphA2-mediated endothelial cell migration is regulated by Rac1 GTPase. (A) Active GTP-bound forms of Rac1 and cdc42 were analyzed by Pak-PBD pulldown followed by immunoblot in lysates from heterozygous (+/-) or EphA2-deficient (-/-) MPMEC stimulated with ephrin-A1. Total Rac1 and cdc42 levels within the lysate prior to PBD-pulldown were detected by immunoblot. Data are representative of four independent experiments. (B) Activation of Rac1 induced by ephrin-A1 stimulation was confirmed in BPMEC, and occurred upon initiation of EphA2 autophosphorylation. (C) Ephrin-A1-induced migration of mock transfected or EphA2-Neu<sup>TM</sup>-expressing BPMEC in the presence or absence of GTPase inhibitor Toxin B was quantified by transwell assay. Significant differences in migration are indicated where  $P < 0.01$  using Student's *t*-test: \* $P = 0.0001$  mock + ephrin-A1 versus mock + ephrin-A1/Toxin B, \*\* $P = 0.0005$  EphA2-Neu<sup>TM</sup> versus EphA2-Neu<sup>TM</sup>/Toxin B. (D) Migration of BPMEC transfected with an expression construct

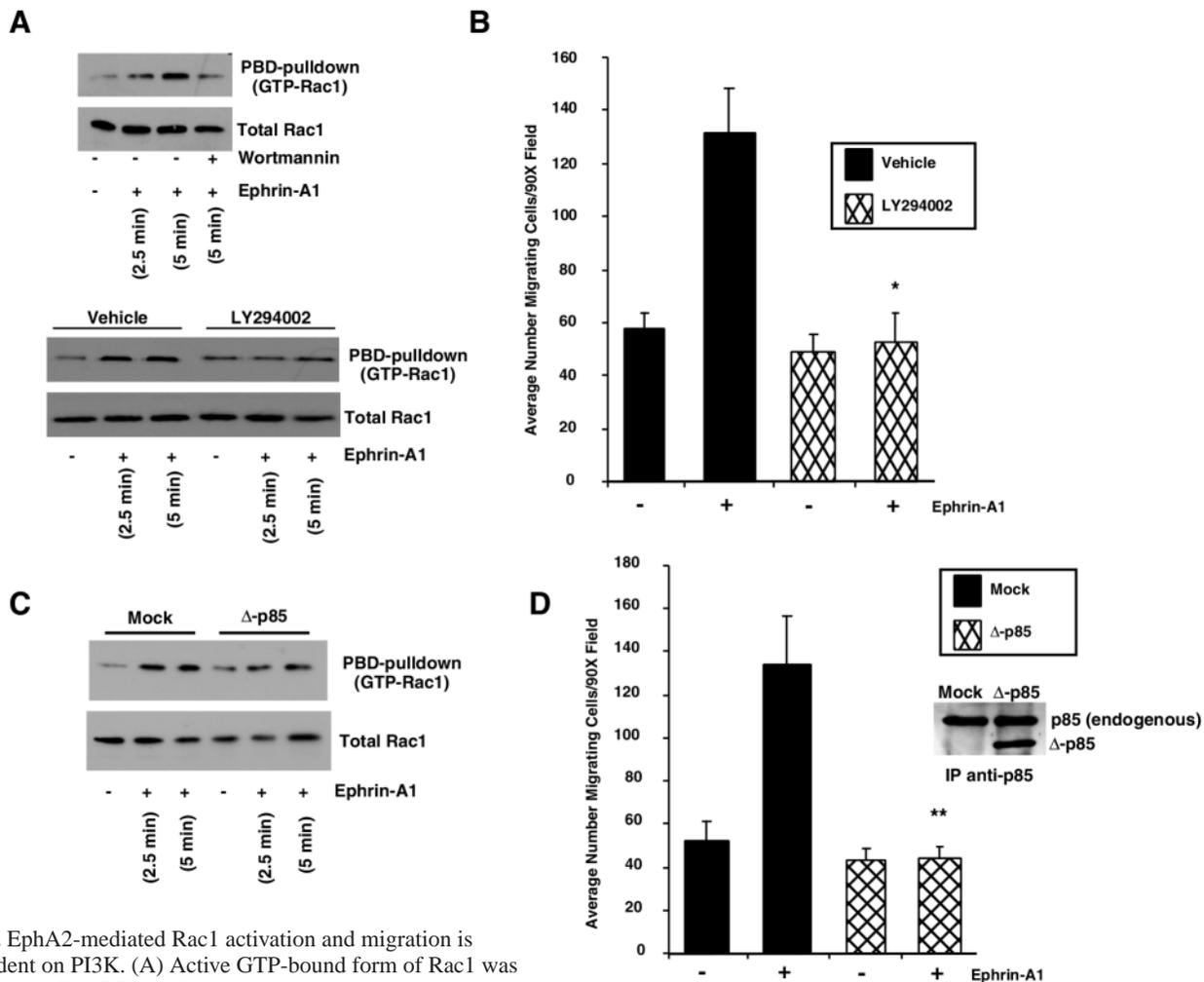
encoding dominant negative Rac1 (Rac1-N17) in response to ephrin-A1 was also quantified by transwell assay by scoring three fields per transwell for each condition in triplicate samples. Data are means  $\pm$  s.d. of three independent experiments. Significant differences in migration are indicated where  $P < 0.01$  using Student's *t*-test: \*\*\* $P = 0.0005$  mock + ephrin-A1 versus Rac1-N17 + ephrin-A1. Expression of Rac1-N17 was confirmed by myc immunoblot.

treated sponges relative to control IgG sponges (Fig. 6A). In contrast, EphA2-deficient mice showed a striking reduction in surface vascular density in response to ephrin-A1-treated sponges. We also observed host vessel penetration into the periphery of ephrin-A1-treated sponges in heterozygous mice (Fig. 6B,C). Though host vessels were observed in the skin surrounding IgG-treated sponges (Fig. 6C), no infiltration of these vessels into these control sponges was detected (Fig. 6B,C). Ephrin-A1-treated sponges collected from EphA2-deficient mice also displayed little infiltration of host vessels

into the sponge. These data suggest that EphA2 RTK is required for ephrin-A1-mediated angiogenic remodeling in vivo.

#### EphA2 deficiency impairs vascular assembly and endothelial cell survival in vivo

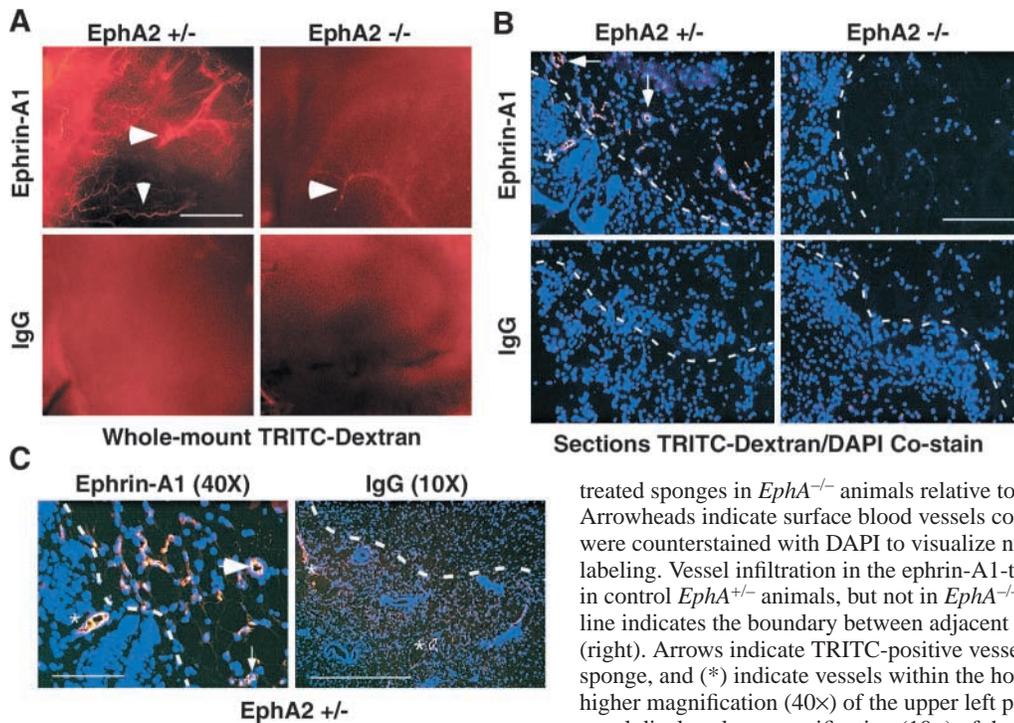
Our in vitro data suggest that the defect in ephrin-A1-mediated angiogenesis in EphA2-deficient mice is probably due to EphA2-deficiency in endothelial cells. Since all host



**Fig. 5.** EphA2-mediated Rac1 activation and migration is dependent on PI3K. (A) Active GTP-bound form of Rac1 was analyzed by Pak-PBD pull-down assay using lysates from ephrin-A1-stimulated BPMEC in the presence or absence of PI3K inhibitors, wortmannin or LY294002. Total Rac1 levels within the lysate prior to PBD-pull-down were detected by immunoblot. Data are representative of three independent experiments. (B) Migration of ephrin-A1-stimulated BPMEC, in the presence or absence of LY294002 PI3K inhibitor, was quantified by transwell assay by scoring three fields per transwell for each condition in triplicate samples. Data are means $\pm$ s.d. of three independent experiments. Significant differences in migration are indicated where  $P < 0.01$  using Student's  $t$ -test: \* $P = 0.0005$  vehicle + ephrin-A1 versus LY294002 + ephrin-A1. (C) Activation of Rac1 was determined by Pak-PBD pull-down assay in BPMEC expressing a dominant negative p85 PI3K regulatory subunit ( $\Delta$ -p85) in response to ephrin-A1 stimulation. (D) Migration of BPMEC transfected with  $\Delta$ -p85 in response to ephrin-A1 was quantified by transwell assay by scoring three fields per transwell for each condition in triplicate samples. Data are means $\pm$ s.d. of three independent experiments. Significant differences in migration are indicated where  $P < 0.01$  using Student's  $t$ -test: \*\* $P = 0.0005$  mock + ephrin-A1 versus  $\Delta$ -p85 + ephrin-A1. Expression of  $\Delta$ -p85 was confirmed by immunoprecipitation of p85 (endogenous and truncated  $\Delta$ -p85) followed by p85 immunoblot.

tissue are deficient for EphA2, however, it is possible that other host cell types contribute to this impaired angiogenic response in null mice. As EphA2 deficiency impairs endothelial cell assembly and migration in vitro, we wished to determine the effect of EphA2 deficiency on endothelial cell assembly in vivo. Previous studies have demonstrated that exogenous endothelial cells will undergo vascular assembly and mimic vessel morphogenesis when immobilized in a solid matrix and transplanted into immunocompromised mice (Nor et al., 2001a; Nor et al., 2001b). We used a similar technique to assess the ability of EphA2-deficient MPMEC to undergo assembly in vivo relative to control MPMEC derived from heterozygous donors. MPMEC were isolated from donor mice and transduced with adenoviruses encoding a  $\beta$ -galactosidase construct in which  $\beta$ -galactosidase expression is targeted to

the cell nucleus. The cells were resuspended in growth-factor-reduced Matrigel and injected subcutaneously into the dorsal flank of nude mice to create a solid 'plug' (Brantley et al., 2002). The plugs were surgically removed after 4, 7 or 10 days, and sections were subjected to X-gal staining to identify exogenous endothelial cells and to score vascular assembly in vivo. Comparable numbers of  $\beta$ gal-positive (LacZ-positive) cells were detected in plugs harboring  $EphA2^{+/-}$  and  $EphA^{-/-}$  MPMEC after 4 days (Fig. 7A). After 7 or 10 days, however, fewer EphA2-deficient MPMEC were detected in the plugs relative to MPMEC derived from heterozygous mice (Fig. 7A,B). The LacZ-positive MPMEC derived from heterozygous mice displayed an elongated cell morphology typical of endothelial cells in vivo, and appeared to be assembled into vessel-like structures (Fig. 7B). In contrast, the



**Fig. 6.** EphA2-deficiency impairs ephrin-A1-induced angiogenesis in vivo. (A) Sponges impregnated with ephrin-A1 or IgG were subcutaneously implanted into the dorsal flank of EphA2 heterozygous (EphA2<sup>+/-</sup>) or EphA2-deficient (EphA2<sup>-/-</sup>) mice. After 7 days, mice were injected intravenously with TRITC-dextran to visualize host blood vessels associated with sponges. Fewer surface vessels were associated with ephrin-A1-

treated sponges in EphA<sup>-/-</sup> animals relative to EphA<sup>+/-</sup> controls. Scale bar, 5 mm. Arrowheads indicate surface blood vessels covering sponges. (B) Sponge sections were counterstained with DAPI to visualize nuclei relative to TRITC vessel labeling. Vessel infiltration in the ephrin-A1-treated sponge periphery was detected in control EphA<sup>+/-</sup> animals, but not in EphA<sup>-/-</sup> animals. Scale bar, 10  $\mu$ m. Dashed line indicates the boundary between adjacent host skin tissue (left) and sponge (right). Arrows indicate TRITC-positive vessels that have infiltrated into the sponge, and (\*) indicate vessels within the host skin tissue. (C) Left panel displays higher magnification (40 $\times$ ) of the upper left panel in B. Scale bar, 5  $\mu$ m. Right panel displays low magnification (10 $\times$ ) of the lower left panel in B, demonstrating

the distance of host vessels relative to the boundary of IgG-treated sponges in EphA<sup>+/-</sup> animals. Scale bar, 1 mm. Data are a representative of results from three independent mice per genotype.

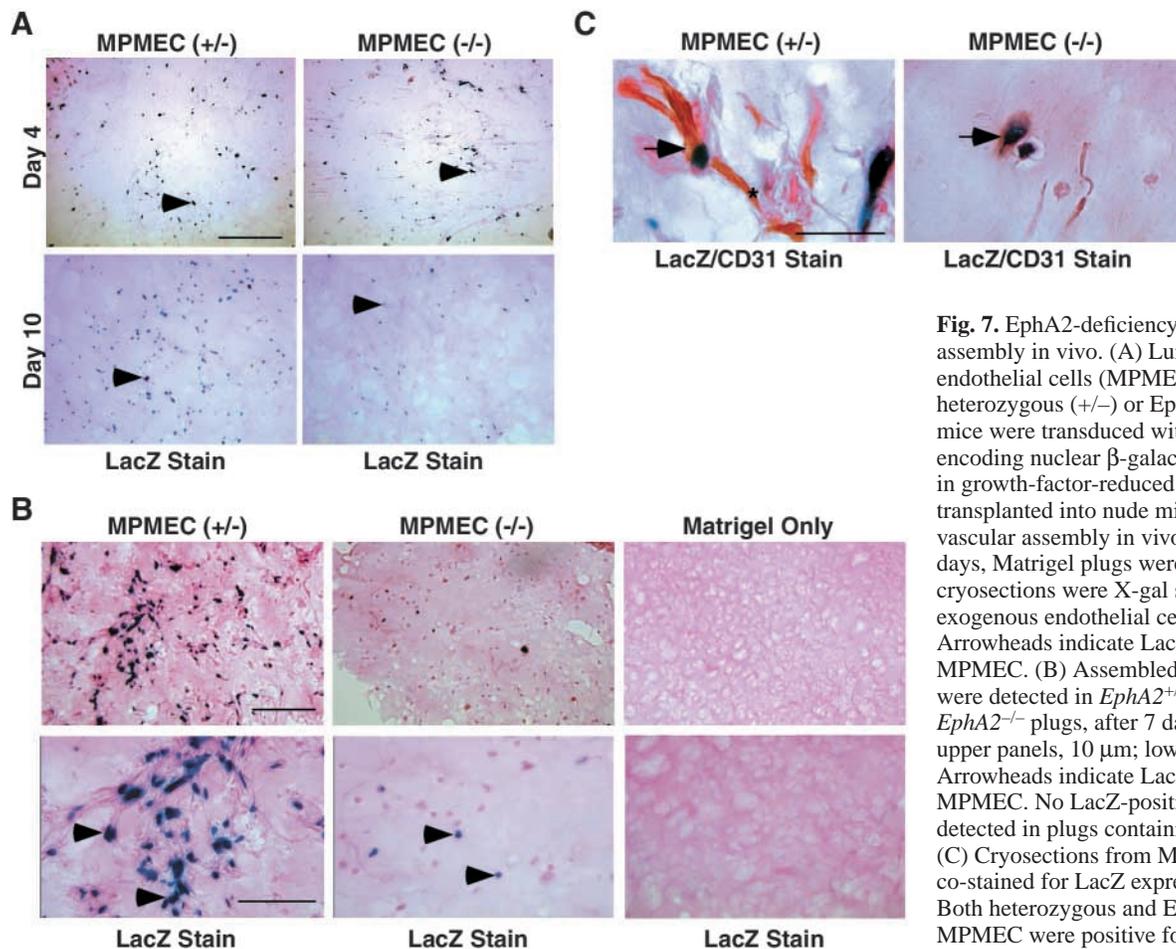
small number of LacZ-positive cells derived from EphA2-deficient mice were rounded in morphology, and did not appear to coalesce and assemble into vessel-like structures (Fig. 7B). While both heterozygous and EphA2-deficient MPMEC retained expression of the endothelial cell-specific marker CD31, EphA2-deficient MPMEC failed to associate with adjacent host endothelium (Fig. 7C). These data suggest that endothelial EphA2 RTK regulates vascular assembly in vivo.

The apparent reduction in the number of EphA2-deficient MPMEC detected in 7 and 10 day plugs may be the result of defects in migration and coalescence such that the cells remained dispersed throughout the plug. Thus, fewer LacZ-positive cells are detected in each field relative to coalescing EphA2<sup>+/-</sup> cells. Alternatively, defects in proliferation and/or survival may also result in decreased MPMEC cell density in EphA2-deficient plugs. To test this hypothesis, we assessed proliferation in LacZ-positive MPMEC by quantifying nuclear expression of Ki67, a marker of proliferation for several cell types, including endothelial cells (Fig. 8A) (Kalashnik et al., 2000) in plugs collected 4 and 10 days post-transplantation. The numbers of LacZ-positive/Ki67-positive nuclei were comparable in EphA2<sup>+/-</sup> versus EphA2<sup>-/-</sup> MPMEC at each time point (Fig. 8A), suggesting that loss of the EphA2 receptor does not affect endothelial cell proliferation in vivo. We also determined apoptosis in LacZ-positive MPMEC by TUNEL analysis. As shown in Fig. 8B, we observed a significant increase in LacZ-positive/TUNEL-positive nuclei in plugs harboring EphA2-deficient endothelial cells versus EphA2<sup>+/-</sup> cells. These data indicate that, in addition to vascular assembly, EphA2 RTK is also required for endothelial cell survival in vivo.

## Discussion

EphA receptors and their ligands of the ephrin-A family transduce short-range signals, regulating endothelial cell migration and assembly during angiogenesis. However, because multiple ephrin-A ligands and EphA receptors can interact promiscuously, the precise A class receptor(s) that function in endothelial cell migration and assembly remains undefined. Indication of involvement of the EphA2 receptor came from studies performed in our laboratory (Cheng et al., 2003; Cheng et al., 2002b) and others (Ogawa et al., 2000), using either an antisense oligonucleotide or expression of a dominant negative EphA2 receptor to inhibit EphA2 function in endothelial cells. In this report, using EphA2-deficient endothelial cells, we demonstrate that EphA2 is a major regulator of angiogenic processes in adult endothelial cells. EphA2-deficient endothelial cells fail to undergo cell migration and vascular assembly in response to ephrin-A1 in vitro. These defects can be rescued by restoring EphA2 expression through adenovirus-mediated transduction. EphA2-deficient mice also display impaired angiogenesis in response to ephrin-A1 stimulation in vivo. Furthermore, while heterozygous control donor endothelial cells can spread, coalesce, and associate with host endothelium in vivo, EphA2-deficient cells display decreased survival and fail to spread and assemble when transplanted into recipient mice. Taken together, these data suggest that EphA2 RTK is required for angiogenic remodeling in mature tissues, particularly in response to ephrin-A1. Because EphA2 deficiency results in a comparable inhibition of endothelial cell migration and assembly to that induced by soluble EphA-Fc receptors (Brantley et al., 2002; Cheng et al., 2002b), the EphA2 receptor is probably a major target.

Investigation of ephrin/Eph receptor-mediated signal



**Fig. 7.** EphA2-deficiency impairs vascular assembly in vivo. (A) Lung microvascular endothelial cells (MPMEC) isolated from heterozygous (+/-) or EphA2-deficient (-/-) mice were transduced with adenoviruses encoding nuclear  $\beta$ -galactosidase, suspended in growth-factor-reduced Matrigel, and transplanted into nude mice to examine vascular assembly in vivo. (A) After 4 and 10 days, Matrigel plugs were collected and cryosections were X-gal stained to detect exogenous endothelial cells. Scale bar, 10  $\mu$ m. Arrowheads indicate LacZ-positive exogenous MPMEC. (B) Assembled vascular structures were detected in *EphA2*<sup>+/-</sup> plugs, but not in *EphA2*<sup>-/-</sup> plugs, after 7 days. Scale bar in upper panels, 10  $\mu$ m; lower panels, 5  $\mu$ m. Arrowheads indicate LacZ-positive exogenous MPMEC. No LacZ-positive cells were detected in plugs containing Matrigel only. (C) Cryosections from MPMEC plugs were co-stained for LacZ expression and CD31. Both heterozygous and EphA2-deficient MPMEC were positive for CD31 staining (arrows), though EphA2-deficient MPMEC

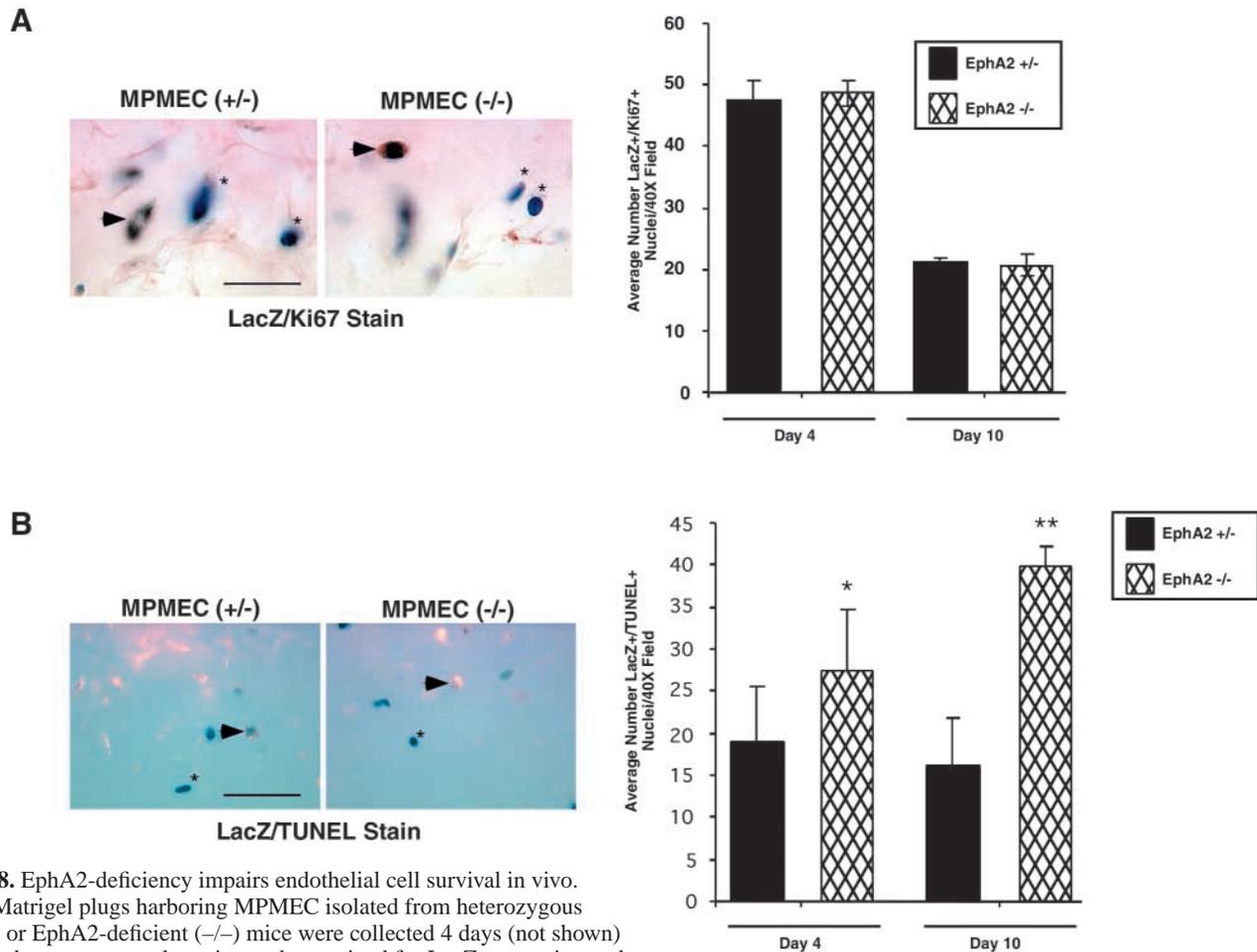
lacked the elongated, endothelial morphology observed in heterozygous MPMEC. LacZ-positive, exogenous MPMEC from heterozygous mice associated with host endothelium (asterisk), while EphA2-deficient MPMEC did not. Scale bar, 2  $\mu$ m. Data are representative of six independent plugs per genotype derived from three independent donor mice from each genotype.

transduction mechanisms that regulate cellular responses in various cell types has been centered on Rho-family GTPases. For example, whereas EphB2 facilitates dendritic spine outgrowth and morphogenesis through activation of *cdc42* in hippocampal neurons (Irie and Yamaguchi, 2002), ephrin-A5 stimulation leads to activation of RhoA and inhibition of Rac1 in cultured retinal ganglion cells, which is accompanied by chemorepulsive axonal growth cone collapse (Wahl et al., 2000). Ephrin-A5 also stimulates cell-rounding and loss of adhesion in EphA3-expressing 293T cells, which involves activation of Rho (Lawrenson et al., 2002). Activation of EphA4 stimulates RhoA activity in developing neurons (Shamah et al., 2001) and vascular smooth muscle cells (Ogita et al., 2003), facilitating growth cone collapse and contractility, respectively. By contrast, activation of EphA4 in *Xenopus* embryos is associated with RhoA inhibition and loss of cell-cell adhesion and polarity (Winning et al., 2002), demonstrating that individual Eph RTKs can activate or repress Rho GTPase signaling in different cell types and processes. These observations illustrate the complexity in Eph-mediated signal transduction and highlight the need to study ephrin-mediated Rho family GTPase responses in specific cell types and physiological contexts.

Deroanne et al., have shown that in the vasculature,

stimulation of vascular smooth muscle cells with ephrin-A1 inhibits cell spreading via inactivation of Rac1 and PAK (Deroanne et al., 2003). In endothelial cells, however, we observed activation of Rac1 and induction of migration in ephrin-A1-treated endothelial cells (Figs 3, 4). This seeming contradiction in activation of Rac1 GTPase between endothelial cells and vascular smooth muscle cells, however, is consistent with functions of respective cell types in the process of angiogenesis. In order for endothelial cells to sprout from pre-existing vessels, the first step required is to relax the interaction between endothelial cells and perivascular supporting cells, allowing endothelial cells to proliferate and migrate towards angiogenic cues. Thus, ephrin-A1 stimulation leads to inhibition of Rac1 activation and cell spreading in smooth vessel cells in the vessel wall to destabilize blood vessels. In contrast, ephrin-A1 activates Rac1 and induces cell migration and vascular assembly in endothelial cells, promoting new capillaries to sprout and branch from pre-existing vessels.

In the present study, we propose a model in which EphA2-mediated endothelial cell migration proceeds through PI3K-mediated activation of Rac1. Several lines of evidence suggest that EphA2 receptor activation modulates Rac1 GTPase



**Fig. 8.** EphA2-deficiency impairs endothelial cell survival in vivo. (A) Matrigel plugs harboring MPMEC isolated from heterozygous (+/-) or EphA2-deficient (-/-) mice were collected 4 days (not shown) or 10 days post-transplantation and co-stained for LacZ expression and the proliferation marker Ki67. Arrows indicate LacZ+/Ki67+ nuclei, and the asterisks indicate LacZ+/Ki67- nuclei. Scale bar, 2.5  $\mu$ m. (Right) The percentage of LacZ+/Ki67+ nuclei relative to total LacZ+ nuclei in each field was calculated to quantify proliferation in exogenous endothelial cells. (B) Matrigel plugs were also subjected to staining for LacZ followed by TUNEL assay to detect apoptosis at 10 days (left). Arrows indicate LacZ+/TUNEL+ nuclei, and the asterisks indicate LacZ+/TUNEL- nuclei. Scale bar, 5  $\mu$ m. (Right) The percentage of LacZ+/TUNEL+ nuclei relative to total LacZ+ nuclei in each field was calculated to quantify apoptosis in exogenous endothelial cells. Data are means  $\pm$  s.d. of three independent samples/condition. Significant differences in percentage of apoptotic nuclei are indicated where  $P < 0.01$  using Student's *t*-test: \* $P = 0.003$  EphA2<sup>+/-</sup> versus EphA2<sup>-/-</sup> 4-day plugs, \*\* $P = 0.03$  EphA2<sup>+/-</sup> versus EphA2<sup>-/-</sup> 10-day plugs.

activity. First, ephrin-A1 stimulation increased levels of GTP-bound active Rac1 proteins, and this increase was blocked in EphA2-deficient endothelial cells, as judged by the PBD-pull down assay. Second, Toxin B, an inhibitor of small GTPases, or a dominant negative mutant of Rac1, Rac1-N17, blocked ephrin-A1-induced cell migration. Finally, inhibition of Rac1 activity by PI3K inhibitors, wortmannin or LY294002, or by a dominant negative  $\Delta$ -p85 mutant, impaired endothelial cell motility in response to ephrin-A1 stimulation. Taken together, these findings suggest that Rac1 plays an important role in EphA2 receptor-mediated endothelial cell migration.

The EphA2 receptor-induced change in Rac1 activity could be regulated by at least two mechanisms. One possibility is that activated EphA2 receptor directly interacts with and changes the phosphorylation state of guanine nucleotide exchange factors (GEFs) and/or GTPase activating proteins (GAPs). For example, EphA4 receptor stimulates RhoA activity in developing neurons through binding directly to ephexin (Shamah et al., 2001), and in vascular smooth muscle cells via direct interaction with Vsm-

RhoGEF (Ogita et al., 2003). GEFs such as Tiam-1 or Vav are potentially interesting candidates that could couple EphA2 RTK to Rac1 activation, as they are expressed in endothelial cells (Georgiades et al., 2002; Lampugnani et al., 2002). Alternatively, GEF activity can also be modified as a result of EphA2-induced regulation of PI3-kinase signaling. This hypothesis is supported by the fact that EphA2 binds the p85 subunit of PI3-kinase (Pandey et al., 1994), and PI3-kinase lipid products have been shown to regulate the activity of GEFs such as Vav and Tiam-1 (Cantley, 2002; Cantrell, 2001). In addition, as shown in Fig. 5, inhibition of PI3-kinase activity by either wortmannin/LY294002 or a dominant negative  $\Delta$ -p85 mutant blocked ephrin-A1-induced Rac1 activation and cell migration. Further studies utilizing specific pharmacologic and dominant-negative reagents, as well as more detailed structure-function analyses, should help to elucidate the mechanism of regulation of Rac1 activation by EphA2 receptor.

Embryonic angiogenesis is regulated by B class Eph receptor signaling, as targeted disruption of EphB2, EphB3,

or ephrin-B2 results in embryonic lethality and vascular remodeling defects (Adams et al., 1999). While ephrin-A1 is expressed in the embryonic vasculature during development, vascular expression of EphA2 is not observed during embryogenesis (McBride and Ruiz, 1998; Ruiz and Robertson, 1994). Moreover, no vascular defects in embryonic angiogenesis have been reported in EphA4 or EphA8-deficient mice (Coonan et al., 2001; Park et al., 1997). It is therefore possible that EphA2 RTK, and perhaps other A class Eph RTKs, may preferentially regulate angiogenesis in adults. Similarly, targeted disruption of other regulators of angiogenesis, such as  $\alpha 1$  integrin and placental growth factor (Carmeliet et al., 2001; Pozzi et al., 2000), result in angiogenic defects in mature animals without affecting embryogenesis, providing precedence for molecular regulation of post-natal, but not developmental, angiogenesis by certain signaling pathways. In addition, EphA2 receptor function may regulate tumor angiogenesis. EphA2-deficient endothelial cells show similar defects in ephrin-A1-induced migration as that elicited by soluble EphA receptor fusion proteins, which also inhibit tumor angiogenesis in vivo (Brantley et al., 2002; Cheng et al., 2002b; Cheng et al., 2003). Thus, these EphA2-deficient mice provide an excellent model system in which to investigate the function of EphA2 in tumor neovascularization.

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