Expression of RAC2 in endothelial cells is required for the postnatal neovascular response

Pradip De*, Qiong Peng*, T. DmitryΦ, Weiming Li, Mervin C. Yoder®, Keith L. MarchΦ, and Donald L. Durden

*Department of Pediatrics, Aflac Cancer Center and Blood Disorders Services, Emory University School of Medicine, Atlanta, GA 30322

ΦHerman B Wells Center for Pediatric Research, Department of Pediatrics, Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, 46202

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Abstract
Herein, we describe an obligate role for the hematopoietic specific GTPase, RAC2 in endothelial integrin signaling and the postnatal neovascularization response in vivo. Using a Rac2 knockout mouse model, we discovered that despite the presence of both RAC1 and RAC2 protein in endothelial cells, RAC2 is obligately required for the postnatal neovascular response and αvβ3/α4β1/α5β1 integrin-directed migration on vitronectin, H296 and CH271, fibronectin fragments, respectively. The molecular basis for RAC2 specificity was explored. A genetic analysis of Syk−/− or Syk−/++; Rac2−/− mice revealed that SYK kinase is required for the integrin induced activation of RAC2. The analysis of endothelial cells from Rac2−/− versus Syk−/++; Rac2−/− mice provided genetic evidence that SYK-RAC2 signaling axis regulates integrin (αvβ3, α4β1 and α5β1) dependent migration. Our results provide evidence that a specific region of the nonreceptor protein tyrosine kinase, SYK, the B linker region containing Y342 and Y346 is required for SYK’s regulation of RAC2 and integrin dependent migration. Moreover, the capacity of mice to vascularize the ischemic hindlimb following femoral artery ligation or matrigel plugs was markedly reduced in mice homozygous deficient for the Rac2 gene. These findings identify a novel signaling axis for the induction and potential modulation of postnatal angiogenesis.

Introduction
The signal transduction events within endothelial cells (EC) which encode the complex cascade of events that are required for angiogenesis are just now coming to light [1]. Angiogenesis was originally defined as the process by which new blood vessels are formed from pre-existing vascular structures. The process is important for certain neovascular events which result in the
reestablishment of a vascular blood supply following ischemia and in tumor-induced angiogenesis [2]. More recent evidence suggests that certain types of neovascularization responses are dependent upon bone marrow derived endothelial cells and/or circulating endothelial precursor cells (CEP or EPC) or hemangiocytes populations, which can be detected resident within mature blood vessels as a source for angiogenic responsiveness [3,4]. More recently, Zengin et al observed that a vascular wall resident endothelial progenitor population of cells is important in postnatal angiogenesis [5]. Regardless of the source of EC, it is likely that a better understanding of the fundamental signaling events responsible for endothelial signal relay will prove useful in the identification of new molecular targets for vascular therapeutics [3]. Remodeling of extracellular matrix and alterations in integrin-mediated adhesion/migration within endothelial cells are required for neovascularization/angiogenesis in vivo [6]. The small guanosine triphosphatases (GTPases) of the Rho family have been shown to participate in these important processes in a number of cell types including endothelial cells [7,8].

The RAC family of small G proteins is composed of three isoforms, RAC1, RAC2 and RAC3 [9]. The RAC1 and RAC3 are ubiquitously expressed whereas RAC2 is selectively expressed in hematopoietic cells [10]. Experiments performed in the Rac2 knockout mouse model have established a prominent role for RAC2 in hematopoietic cells including neutrophil, macrophage and mast cell defects [11–13]. Since hematopoietic cells and endothelial cells are both derived postnatally from the same bone marrow compartment and share the expression of certain common signaling proteins, we hypothesized that hematopoietic specific small GTPase, RAC2 may play a role in endothelial cells and hence may be an important signaling pathways in the control angiogenesis.

To test this hypothesis, we utilized a Rac2 knockout mouse model to examine the role of Rac2 loss in endothelial cell function(s) and angiogenesis. Herein, we demonstrate RAC2 expression in endothelial cells and provide direct evidence that this small G protein is required for integrin (αβ3, α4β1 and α5β1) directed migration of endothelial cells and the angiogenic response in vivo. Results from analysis of mouse genetic models (Syk−/+, Syk+/+; Rac2−/+) provide evidence that SYK kinase is required for the activation of RAC2 and endothelial cell migration via the αβ3 integrin. Moreover, using a reductionistic approach in COS7 cell (transfection with B linker region SYK mutations at Y317F, Y342F, Y346F and catalytically dead SYK, K396R) generate additional evidence that SYK can selectively mediate the activation of RAC2 downstream of a4β1 integrin engagement and SYK can augment RAC2-dependent migration via this integrin. Moreover, we have identified a subregion of SYK, the B linker sequence, required for the activation of RAC2 and cell migration. These combined observations suggest that SYK-RAC2 signaling axis and specifically the B linker region of SYK are new molecular targets for the regulation of neovascular/angiogenic response of endothelial cells in vivo.

Considering the importance of postnatal neovascularization, the identification of a novel molecular pathway, SYK-RAC2 signaling axis in endothelial cells that controls post embryonic angiogenesis may result in the development of innovative therapeutic strategies (e.g. RAC2 or SYK inhibitors).

**Materials and Methods**

**Animals, antibodies and reagents**

Rac2 knockout and Syk haploinsufficient (−/+ ) mice and normal littermates in C57BL/6J genetic background have been described [11,14,15]. We adhere to Emory University Animal Care and Use Committee approved protocols and NIH guidelines. The FVN/N-TgN (Tie2-GFP) transgenic mice were obtained from commercial stocks at the Jackson Laboratory (Bar Harbor, ME). Human brain endothelial cells (HBEC) were obtained as described [16]. The HUVEC, J774 and COS7 cells were purchased from ATCC (Rockville, MD). RAC2 Ab was
a gift from Drs. G. Bokoch and U. Knaus (The Scripps Research Institute, La Jolla, CA). PAK-1 PBD (RAC/CDC42 assay reagent), agarose for pull down of the activated RAC1 and RAC2 and monoclonal RAC1 antibody were from Upstate Biotechnology (Lake Placid, NY). Recombinant human fibronectin peptides, H296 and CH271 were obtained from Collaborative Biomedical (Bedford, MA) or were a gift from Takara Shuzo (Otsu, Japan). Vitronectin was purified as described [17]. GM-CSF was bought from Peprotech (Rocky Hill, NJ). Dil acetylated low-density lipoprotein (Ac-LDL) and vWF-conjugated FITC rat anti-mouse mAbs were procured from Molecular probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

**Isolation of bone marrow derived endothelial cells (BMEC)**

Bone marrow derived endothelial cells (BMEC) were generated by flushing the marrow cavity with culture media (DMEM, Life Technologies, Rockville, MD) followed by a separation on histopaque199 (Sigma, St. Louis, MO). Cells were collected, washed and resuspended in DMEM supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin and plated on 10 cm dish and cultured for 24 hours at 37°C (5% CO₂). The next day, media containing nonadherent cells was collected, spun at 1200 rpm and plated on 60 mm dish. After 48 hours culture in 5% CO₂ incubator, nonadherent cells were collected, spun at 1200 rpm, re-suspended in complete media containing 400 ng/ml GM-CSF and cultured for 5–7 days in 5% CO₂/5% O₂ incubator at 37°C.

**Immunolabelling and Dil-labeled acetylated-LDL uptake assay**

For staining, cells grown on tissue culture dishes were washed with PBS, fixed with 4% paraformaldehyde at 4°C for 30 minutes and washed three times with PBS. The cells were then immersed in PBS containing 3% milk, 0.025% Triton X 100 (PMT solution) for 30 minutes to block nonspecific protein interactions and permeabilize the cell membranes. The cells were stained with anti vWF mouse antibody and FITC rat anti-mouse Abs in PMT solution and incubated at 4°C for overnight. Macrophages and appropriate isotype antibodies (IgG2a) were used as a negative control. For uptake of Dil-labeled ac-LDL, cells were incubated with 10 μg/ml Dil-labeled ac-LDL (Biomedical Technologies, Stoughton, MA) for 4 hours at 37°C. After incubation, cells were washed three-times with PBS, fixed with 4% paraformaldehyde for 30 minutes and visualized with a fluorescent microscope (Nikon). We utilized the Tie2-EGFP transgenic mouse model where the Tie2 promoter elements drive the endothelial cell specific expression of green fluorescent protein [18] to visualize and isolate EGFP positive endothelial cells from different organs e.g. heart, lung, kidney and brain in vivo. Cell suspensions from different tissues were prepared as described [19] and subjected to FACS sorting to isolate a highly purified population of EGFP positive cells for further analysis.

**Immunoblot analysis of RAC2 expression**

Lysates of bone marrow derived endothelial cells (BMEC), macrophages (BMM₀), mouse macrophage cell line (J774 A.1), human brain endothelial cell line (HBEC), human umbilical vein endothelial cells (HUVEC), EGFP sorted cardiac endothelial cells from Tie2 transgenic mice and mouse embryo fibroblast (MEF), bone marrow derived macrophages from Rac2 deficient mice (both for negative control) were prepared and protein was estimated with Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. Protein lysates were resolved by 15% SDS PAGE. Blots were probed with rabbit polyclonal Ab for RAC2 and developed with ECL method (Amersham-Pharmacia Biotech, UK).

**RT-PCR analysis**

We evaluated the bone marrow derived endothelial cells, Tie2-GFP positive cells derived from the adult heart and compared them to adult marrow Sca-1 positive/c-kit positive/lin negative
cells using a spectrum of gene transcripts specific for hematopoietic or endothelial lineage cells. Total RNA was isolated for reverse transcription-polymerase chain reaction (RT-PCR) as described earlier by Li et al [19]. PCR products were amplified using oligonucleotide primers previously described [19] and visualized with ethidium bromide in 2% agarose gel. Hematopoietic markers include: βH1 (embryonic hemoglobin), β major (adult hemoglobin), Gata1, Scl, Pu.1 and CD31. Endothelial markers were: collagen type 4, hevin, Flk1, vascular endothelial cadherin (VE-Cadherin, CD144), CD31, insulin-like growth factor binding protein 3 (Igfbp), Tie2 and von Willebrand factor (vWF).

**Integrin directed RAC2 activity**

Primate endothelial cells (passage #4) were isolated as previously described [20]. Cells (primate endothelial cells) or bone marrow derived endothelial cells either from WT, Rac2−/−, Syk−/+;Syk−/++;Rac2−/+/ were stimulated in 10 cm non-tissue culture Petri dishes coated with 20 μg/ml either vitronectin or fibronectin. Cells were washed with serum free medium (3 × 10⁶ cells/2 ml media) and then plated onto vitronectin or fibronectin coated plates for 15 and 30 minutes. Cell lysates were prepared in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM sodium fluoride and 1 mM sodium orthovanadate. For pull-down experiments, clarified lysates were assayed for total protein (Bio-Rad Protein Assay Kit) using BSA as standards. The lysates were used for RAC2 pull-down assays after protein equilibration. Binding reaction was initiated by adding 10 μl of Pak-1 agarose (GST fusion protein, corresponding to the p21 binding CRIIB domain, PBD, residues 67–150, of human Pak-1, expressed in E. coli and bound to glutathione agarose) to each sample and incubated for 45 minutes at 4°C with gentle rocking and processed as described before. GST fusion protein bound proteins and cell lysates were analyzed by Western blot for RAC2-GTP (activated RAC2) and total RAC2 respectively.

**Cell migration assay**

Integrin-directed cell migration assays (haptotaxis) were performed on polycarbonate membranes using transwell migration chamber (diameter 6.5 mm, pore size 8 μm; Costar Corporation, Cambridge, MA). The haptotaxis assay is a quantitative measure of integrin dependent migration [21]. The underside of the membrane to which cells migrate was coated with 20 μg/ml vitronectin, fibronectin or fragments of fibronectin H296 (binds with α5β1) or CH271 (binds with α5β1) in PBS for 1 hour at 37°C. Surfaces were subsequently blocked with heat denatured BSA. Transwells were placed into the lower chamber containing 600 μl serum free media. 2 × 10⁵ cells in 100 μl media/transwell were added to the top of the migration chamber (uncoated side) and allowed to migrate to the coated side of the chamber for 4 hours at 37°C. Haptotaxis was quantified as described [12]. The haptotaxis response was further confirmed by demonstrating the lack of migration when both sides of the membrane were coated with vitronectin, H296 or CH271.

**Preparation of three dimensional aortic ring cultures**

Angiogenesis was studied by culturing rings excised from the mouse aorta in matrigel (BD Biosciences, Discovery Labware, San Diego, CA) with some modifications of the method originally reported for the rat aorta [22]. Thoracic aortas were removed either from wild type or from Rac2 knockout mice following CO2 euthanasia and immediately transferred to culture dish containing ice-cold serum free media (DMEM, Life Technologies). The peri-aortic fibroadipose tissue was carefully removed with fine micro-dissecting forceps and iridectomy scissors paying special attention not to damage aortic wall. 1–2 millimeter long, aortic rings (approximately 10–15 per aorta) were sectioned and extensively rinsed in 5 consecutive washes of DMEM. Ring shaped explants of mouse aorta were then placed between the two layers of 100 μl of growth factor depleted matrigel and overlaid with 200 μl of DMEM. Method for
quantitation of endothelial sprouts is as described by Nicosia and Ottinetti [22]. Briefly, this involves the visual counting of microvessel branches using a 4x objective. Microvessel structures extending outward from the aortic ring are counted. Each branch point of an endothelial structure is counted as a new vessel in replicates of 5 rings evaluated per mouse from each aorta to obtain statistical comparisons.

Hindlimb ischemia model

Wild type (C57BL/J6 female mice) and Rac2 knockout mice, 9–11 weeks of age were used for experiments. All protocols were approved by Indiana University Animal Care and Use Committee and were used on NIH laboratory standards. The animals were anesthetized with 2.5% 2, 2, 2-tribromoethanol (1.5 ml/kg, I.P. Sigma), after which incision was performed on the skin overlying the middle portion of the left hindlimb. The left femoral artery was ligated immediately distal to the inguinal ligament and proximal to the saphenus and popliteal arteries. Excision of the femoral artery occurred after ligating the branches [23]. The femoral artery was excised after ligation of most of its branches. Evaluation of blood flow restoration during the first month was accomplished by utilizing Laser Doppler Imaging system (Moor Instruments) as described by others [24]. The hindlimb perfusion measurements were performed under 1.5% isoflurane gas anesthesia. The animals were placed in a ventral position on a heating pad at 37°C for 5 minutes prior to measurements to minimize temperature variation. Scans were made from planter surface of the foot. Each area of interest was scanned in triplicate and a quantitative analysis was performed by calculating the average ratio of perfusion observed in the ischemic versus control extremity.

Matrigel in vivo angiogenesis model

Growth factor depleted matrigel (400 ul/mouse) supplemented with 600 ng/ml basic FGF (Peprotech, Rocky Hill, NJ) was injected subcutaneously into wild type or Rac2 −/− mice. After 5 days, mice were sacrificed and plugs were evaluated macroscopically, biochemically (VEGFR2/FLK-1 expression) and immunohistochemically for CD31 microvessel staining. Antibody stained cells within matrigel plug were visualized using a peroxidase conjugated anti-rat secondary and hematoxylin counterstaining. A negative control with secondary alone was carried out for each individual plug from each mouse.

Heterologous COS7 migration system

COS7 cells were plated at 1 × 10⁶ cells on a 10 cm-tissue culture dishes overnight. Cells were transiently transfected with plasmids using the Lipofectamine reagent. Episomal plasmids were used to express RAC2, wild type SYK-EGFP tagged or, catalytically dead SYK-EGFP (KD-SYK), Y317F SYK-EGFP, Y342F/Y346F SYK-EGFP, or Y317F/Y342F/Y346F SYK-EGFP. After 4 hours medium was changed and cells were further incubated for 48 hours. For the migration assay, the underside of the polycarbonate membrane of the Transwell was coated with 20 μg/ml of specific ligand (H296). 2 × 10⁵ cells in 100 μl of media were added to the upper part of the well (non-coated side) and allowed to migrate for 4 hours at 37°C. For RAC2 activation assay, cells were transfected with the same plasmid as described above. After 48 hours of transfection, cells were stimulated on 10 cm of non-tissue culture coated Petri dishes coated with 20 μg/ml H296 peptide (a4β1) for 15 minutes at 37°C and the activation assay was performed as described earlier. For all transfections, the plasmid composition and DNA concentrations were identical for all experimental groups. Expression of RAC2 and total RAC were quantified with Western blot.

In vivo knockdown of RAC1 and RAC2 by mouse specific siRNA

RAC1 and RAC2 were knocked down in bone marrow derived endothelial cells by transient transfection of mouse RAC1 and RAC2 specific siRNAs using lipofectamine 2000 following
the manufacturer’s instructions. Transfected cells were harvested and analyzed for the expression levels of RAC1 and RAC2 at 24 and 48 hours. For integrin directed migration assays, mouse specific RAC1 and RAC2 siRNAs were transfected in separate experiments as described above. After 24 and 48 hours of transfection, cells were harvested and migration assay was performed using Transwell chamber (as described above).

Results

RAC2 is expressed in endothelial cells

It has been shown that RAC2 is the predominant RAC isoform in hematopoietic cells [9]. To evaluate the potential involvement of RAC2 in endothelial cells and hence angiogenesis, we examined RAC2 expression in endothelial cells derived from several sources. We prepared bone marrow-derived endothelial cells (BMEC), human brain-derived endothelial cells (HBEC) and human umbilical vein-derived endothelial cells (HUVEC) [16]. To confirm that the bone marrow derived endothelial cells we isolated are of the endothelial lineage; they were examined for the ability to incorporate Dil-ac-LDL along with immunostaining of vWF. The vWF is expressed only in endothelial cells and megakaryocytic cells (Fig. 1A) [25,26] and Dil-ac-LDL is exclusively taken up by endothelial cells and macrophages [26,27]. The results demonstrate that our culture conditions give rise to cells that express vWF and incorporate ac-LDL. Endothelial gene expression profile of bone marrow endothelial cells were also analyzed by RT-PCR for mRNA expression patterns of several endothelial specific transcripts (CD144-VE cadherin, CD31, Tie 2, vWF and Flk1) from 4 separate (lanes 1–4) endothelial cell populations isolated from bone marrow (Fig. 1B). Based on these results, we conclude that the bone marrow derived cells in our experiments are endothelial cells. Finally, we sorted GFP positive EC from several tissues within the Tie2-EGFP transgenic mouse model as described (Fig. 1C) [19]. To further characterize the endothelial and hematopoietic cell populations from the Tie2-EGFP transgenic model (Fig. 1D), we performed RT-PCR analysis on total RNA samples obtained from cells sorted from Tie2-EGFP mice. These included a total of 18 transcripts, 9 were endothelial specific and 9 were specific for hematopoietic cells [19] (Fig. 1D). RT-PCR data confirms cells derived from Tie2-EGFP mice to be endothelial in lineage. Levels of RAC2 protein were compared to hematopoietic cells from different sources, a mouse macrophage cell line, bone marrow derived macrophages from Rac2+/+ mice (as positive control), mouse embryo fibroblasts, MEF and bone marrow derived macrophages from Rac2−/− mice (as negative controls). Quantitation of RAC2 expression was via Western blot analysis with a RAC2 specific antibody as described [10] (Fig. 1E). The RAC2 protein was detected in a mouse macrophage cell line (J774 A.1), bone marrow derived macrophages from Rac2 wild type mice and endothelial cells from other sources. A non-endothelial source from same mouse strain, MEFs was negative for RAC2 protein expression (Fig. 1E).

RAC2 is required for integrin-directed migration in bone marrow derived endothelial cells

We determined the levels of RAC1 and RAC2 protein in mouse bone marrow-derived endothelial cells and MEFs from WT and Rac2−/− mice by immunoblotting with specific antibodies for RAC1 and RAC2 (Fig 2B). RAC2 was only expressed in BMEC, whereas similar amount of RAC1 was detected in BMEC and MEF (Fig. 2B). Previous experiments by Roberts et al have determined that the faint band noted in Fig. 2B, lane 4 (10 μg) is the result of a minor cross reactivity between the Rac2 antibody and the highly conserved Rac1 protein present in the lysate of Rac2−/− cells. As previously described [11], we have confirmed that our Rac2−/− mice do not express mRNA for the Rac2 protein and our routine genotype analysis of each animal studied confirms the existence of the PGK-neomycin insert within the exon 1 of the Rac2 coding sequence in one or both alleles. Previous work from our laboratory determined that RAC2 was required for macrophage integrin-directed cell migration. To determine whether RAC2 is necessary for endothelial integrin αvβ3/αvβ5, a4β1 and a5β1 receptor

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signaling, we evaluated the effects of RAC2 deficiency on bone marrow derived endothelial cells (BMEC) migration on the extracellular matrix (ECM) proteins, vitronectin (VN) or fragments of fibronectin, H296 and CH271, respectively. BMEC adhesion to vitronectin depends on integrin αvβ3/αvβ5, whereas adhesion to H296 and CH271 are predominantly mediated by integrin αβ1 and αβ1, respectively. RAC2 deficiency had no effect on endothelial cell adhesion to these matrix proteins (not shown). In contrast, Rac2−/− endothelial cells display markedly reduced migration via the αvβ3/αvβ5 integrins (p <0.005) (Fig. 2A). Moreover, αβ1 dependent migration (p < 0.001), αβ1 dependent migration (p <0.05) were markedly reduced in Rac2 −/− cells compared to BMEC isolated from Rac2 wild type mice (Fig. 2). Interestingly, migration of BMEC from Rac2 wild type mice on FN-H296 was higher compared to other ligands. Similar results were previously observed in bone marrow derived macrophages from Rac2 wild type mice [12]. The dependence of integrin directed migration of BMEC on Rac2 was further evaluated using mouse-specific siRNAs. To perform these experiments we used specific siRNAs for RAC1 (mouse) and specific siRNA for RAC2 (mouse). Figure 2 shows that BMEC transfected with Rac2 siRNA display marked inhibition of migration (p<0.005) on vitronectin (αvβ3/αvβ5) as compared to Rac1 siRNA transfected cells (Fig. 2C and D). Taken together, we conclude that bone marrow derived endothelial cells require RAC2 for integrin-directed haptotaxis particularly via the α4β1 and αv integrins. Data from siRNA studies suggest that RAC1 is not required for bone marrow derived endothelial cell migration on vitronectin. Furthermore, siRNA experiments suggest that migration is not controlled by the total quantity of RAC protein expressed in BMEC. These integrins are present in blood vessels/endothelial cells and they utilize RAC2 for endothelial cell motility, which is a critical early step in neovascularization/angiogenesis.

**Aortic ring outgrowth response is absent in Rac2−/− mice**

As a measure of *in vitro* angiogenesis and endothelial response we compared the outgrowth of endothelial cell sprouts from aortic rings of Rac2−/− versus Rac2 +/+ mice. Aortic rings were excised and carefully embedded in growth factor depleted matrigel. Aortic rings cultured from Rac2 +/+ mice showed a rapid and robust vessel outgrowth response within 7 days (Fig. 3A). In sharp contrast, the generation of endothelial sprouts from Rac2 −/− mouse aortas was not detectable. The number of sprouts from the cultured aortic rings was quantitated (Fig. 3B). These results suggest a marked defect in the endothelial cell population resident within the Rac2 −/− mature vessel to form endothelial sprouts, an *in vitro* index of angiogenic activity.

**Role of RAC2 on neovascularization of the ischemic hindlimb in vivo**

Hindlimb reperfusion following ischemic challenge was measured with a Laser Doppler Perfusion Imaging (LDPI) system. Blood flow in the ischemic hind limb (leg and foot) was scanned using LDPI scanning. Perfusion of the ischemic hindlimb in mice assessed by doppler measurement (Fig. 4A and B) was markedly reduced in Rac2 −/− animals. On day 4 after surgery, doppler flows were significantly reduced in Rac2 −/− mice and over the subsequent 21 days, limb perfusions remain markedly depressed. We evaluated perfusion of both the left (nonligated, control) and right (ligated) hindlimb of these mice. This non-invasive technique allows us to assess blood flow at multiple time points following arterial ligation. Blood perfusion recovery was measured bi-weekly and body temperature was maintained by keeping animals on a heating pad. By 7 days after surgery, quantitative analysis showed a significant increase in blood flow in Rac2 +/+ mice. In marked contrast, a significant reduction in revascularization is observed in the Rac2 −/− animals.

**Neovascularization of matrigel plugs in vivo**

To further evaluate the role of RAC2 in the neovascular response (in the absence of vascular stress, injury or inflammation), we studied bFGF-induced vascularization using *in vivo* matrigel...
plug angiogenesis model (Fig. 5). Consistent with the observed defect in Rac2−/− mice (as studied in ischemic hindlimb model, Fig. 4), Rac2−/− mice are markedly defective in the bFGF-matrigel induced neovascular response as measured by density of the CD31 staining of endothelial vessels or by Western blot analysis for the endothelial specific marker KDR. These results provide additional evidence that RAC2 functions in signaling pathways required for the neovascular response in vivo.

Vitronectin and fibronectin differentially activate RAC2 in primate endothelial cells
To further support a role for RAC2 in EC, we tested the extent to which integrin specific engagement leads to activation of RAC2 in primary endothelial cells isolated from a nonhuman primate. The baboon-derived endothelial cells were characterized morphologically and by immunohistochemical staining (positive for VE-cadherin, vWF and CD31) as described [20]. These endothelial cells express RAC2 and following adhesion to extracellular matrix proteins (vitronectin and fibronectin) activate this GTPase. The most marked activation of RAC2 in these ECs is observed following fibronectin stimulation a response that directly correlates with the higher magnitude of migration on fibronectin as compared to vitronectin. Interestingly, RAC1 activation occurs following integrin (either vitronectin or fibronectin) stimulation (Fig. 6A, lower panel) but this activation does not correlate with migration response in these ECs. Our data are consistent with the fact that RAC2 is present in another primary endothelial cell source (nonhuman primate endothelial cells) and plays a role in a response required for angiogenesis (integrin-directed migration on provisional matrix proteins) (Fig. 6).

Role SYK kinase on α4β1/αvβ3 dependent migration and activation of GTPase, RAC2
To explore the molecular basis for RAC2 specificity in endothelial cells signaling we utilized an heterologous COS7 cell expression system where we can control expression of RAC2 and SYK kinase proteins not normally expressed in COS7 cells. COS7 cells were cotransfected with plasmids coding for RAC2 and/or SYK or catalytically dead SYK or mutant forms of SYK lacking one (Y317F) or more (Y317F/Y342F and Y317F/Y342F/Y346F) linker region tyrosine phosphorylation sites. All forms of SYK were tagged with EGFP to control for transfection efficiency. The percentage of cells expressing SYK-EGFP and intensity of fluorescence was analyzed by flow cytometry as described earlier [12]. We have previously demonstrated that SYK is necessary for RAC2 dependent migration in COS cells on H296. As shown in Figure 7, integrin directed COS7 cell migration was dependent on wild type SYK. Cells transfected with either RAC2 alone or catalytically dead SYK (KD-SYK) or WT-SYK alone failed to migrate on H296. Interestingly, a mutant form of SYK lacking the tyrosine residues at position 342 and 346 or lacking all three linker tyrosine phosphorylation sites at Y317, Y342 and Y346 were defective in their capacity to induce α4β1 dependent migration and α4β1 dependent activation of RAC2 (Fig. 7A, B and C). Thus, in this model system, SYK is capable of specifically activating RAC2 and not RAC1 [12] and specific tyrosine phosphorylation sites within the linker region of SYK i.e. Y342F/Y346F and Y317F/Y342F/Y346F are required to promote RAC2 activation and integrin (α4β1) dependent cell migration. We and others have observed SYK kinase expression in endothelial cells [28,29]. In order to validate our observation that SYK kinase is a necessary upstream regulator of RAC2 function in BMEC, we used a genetic approach. Mature BMEC from WT or Syk−/+ mice or Syk−/−; Rac2−/+ (double heterozygote) mice were tested for integrin (αvβ3αvβ5) directed migration and activation of RAC2. The data show that both integrin directed migration and activation of RAC2 are markedly reduced in BMEC from Syk−/+ mice as compared to BMEC from wild type mice (Fig. 8A and B). Integrin directed migration and activation of RAC2 are further decreased by the loss of one allele of Syk and one allele of Rac2 in BMEC (Syk−/+; Rac2−/+)(Fig. 8A and B). In contrast, augmentation of RAC1 activity following integrin stimulation was not affected in bone marrow endothelial cells generated either from Syk−/+ mice or Syk−/−;Rac2−/+ mice (Fig. 8B, lower panel). These data suggest that RAC2 is a unique
downstream target for SYK kinase and SYK-RAC2 signaling axis is required for integrin directed BMEC migration on vitronectin. These combined data suggest a molecular basis by which hematopoietic and endothelial cells may activate RAC2 via the SYK-RAC2 signaling axis, and specifically the SYK linker region Y342/Y346 sites. We hypothesize that the SYK-RAC2 axis, potentially via the activation of VAV1 or PLC\(\gamma\)1 are required for important endothelial functions like av\(\beta\)3 and a4\(\beta\)1 integrin signaling, migration and neovascularization/angiogenesis.

Discussion

The induction and subsequent downregulation of the neovascular response involves a complex array of signaling events executed via different integrins (av\(\beta\)3/av\(\beta\)5/a5b1) and growth factors (VEGF, bFGF) carefully orchestrated over time and space [30]. A good example of the complexity is born out in the function of av, \(\beta\)3 and \(\beta\)5 integrin subunits now appreciated from the targeted disruption of these integrin subunits in mice [31] where the \(\beta\)3 integrin subunit appears to play a role in both the upregulation and downregulation of the angiogenic response in vivo [32]. Hence, we hypothesized that the provisional integrins, av\(\beta\)3, av\(\beta\)5, a4\(\beta\)1and a5b1 may partly execute control over neovascularization via a specific signaling circuit expressed selectively in endothelial cells involving a unique set of signaling proteins separate from the nonprovisional integrins expressed on quiescent ECs e.g. a2b1, a1b1, collagen receptors. To examine this hypothesis, we studied endothelial cells and angiogenesis in two knockout mouse models where the Rac2 and Syk genes were deleted separately or in combination [11,33]. Our results demonstrate an obligate role for the monomeric G protein RAC2 and an upstream nonreceptor protein tyrosine kinase, SYK in endothelial integrin signaling, migration and angiogenesis in vivo.

Why would endothelial cells evolve a requirement for the blood specific G protein, RAC2 and SYK for vascular functions? The answer may lie in the rapid and complex environment in which the angiogenic endothelial cell functions. During the initial phase of angiogenesis, endothelial cells (EC) are recruited from the neighboring pre-existing capillaries and/or are recruited from bone marrow derived endothelial precursor cells [34]. Endothelial cells must interact with one another, coordinating vessel integrity, identity, growth and remodeling via integrin av\(\beta\)3 [30,35] and VE-cadherin [36,37]. Therefore both endothelial support cells and inter-endothelial communication are required for proper vessel assembly [38,39]. Moreover, there is considerable evidence that bone marrow derived endothelial cells and endothelial precursor cells (EPC or CEP) are both necessary to mediate a neovascular response in vivo [34]. A recent report by Sebzda et al demonstrated that the combined genetic knockout of SLP-76 and Syk resulted in a vascular defect characterized by abnormal blood lymphatic endothelial connections. This phenotype could be corrected postnatally by the transgenic expression of SLP-76 in a subset of GATA1 positive hematopoietic cells characterized as endothelial precursors in a cell autonomous manner. These results are consistent with our results and suggest a model in which the av\(\beta\)3-SYK-SLP-76-RAC2 signaling axis is important for vascular endothelial cell function and in neovascularization [40,41]. Our results differ from the Syk-SLP76 phenotype in that Rac2 loss is not associated with an embryonic vascular defect and is only associated with alteration in the postnatal angiogenic response in vivo.

A goal of this study was to identify the upstream regulatory molecules (in this case SYK) for the control of RAC2 in EC. The SYK kinase contains two tandem SH2 domains followed by a linker region termed, the B linker region, and C terminal catalytic domain. The B linker region of SYK corresponds to amino acid residues 259 to 364 positioned between the end of the C terminal SH2 domain and the beginning of the catalytic domain. The SYK linker region contains a number of important tyrosine phosphorylation sites including Y317, Y342 and Y346 the conserved binding sites for CBL, VAV or PLC\(\gamma\), respectively [42]. Other work has...
demonstrated that a certain VAV isoform, VAV1 is more efficient as compared to VAV2 or VAV3 in the activation of RAC2 in neutrophil lineage. Recent evidence also suggests that PLCγ1 is required for actin polymerization and hence migration via a downstream effect on the PI-3K cascade [43]. To directly investigate the role played by SYK kinase, we employed a reductionistic strategy using COS7 cells transiently transfected with different mutants of SYK at B linker region (Y317F or Y342F/Y346F or Y317F/Y342F/Y346F) versus catalytically dead SYK (KD-SYK) and WT-SYK to assess their capacity to activate RAC2 following integrin engagement. Under these conditions, Syk transfection does not result in integrin induced activation of RAC1. Our results provide the first evidence that tyrosine residues Y342 and Y346 within insert region of the SYK kinase are necessary for RAC2 dependent integrin-directed migration (Fig. 7). Since these regions of SYK have been observed to mediate an interaction between the β1 and β3 integrins and bind VAV and PLCγ it is possible that SYK’s capacity to activate RAC2 downstream of these integrin receptors may map to one or both of these effectors mechanisms. In contrast, the Y317, a CBL adapter protein-binding site is not required for SYK to interact with RAC2 to drive haptotaxis via the α4β1 integrin (Fig. 7).

Results generated in our laboratory demonstrate that the catalytically dead SYK kinase (KD-SYK, K396R) or mutant forms of SYK (Y342F/Y346F and Y317F/Y342F/Y346F) are defective in the induction of RAC2 activation and RAC2-specific migration in COS7 cells (upon α4β1 engagement). These results suggest an important role for linker domain tyrosine phosphorylation sites of SYK (Y342 and Y346) in SYKs interaction with the hematopoietic specific GTPase, RAC2. Furthermore, data generated using BMEC from Syk−/− and Syk−/−;Rac2−/− mice support the notion that SYK is necessary upstream for RAC2 activity (following integrin engagement) which is required for integrin directed migration in BMEC (Fig. 8A and 8B). Recently, Gevrey et al have reported that CX3CL1-induced macrophage migration is dependent on tyrosine phosphorylation of SYK [44]. Interestingly, RAC1 activation was not affected following integrin stimulation in BMEC either from Rac2−/− or Syk−/−;Rac2−/− animals (Fig. 8B lower panel). These data suggest that αvβ3/αvβ5 dependent activation of RAC1 in BMEC is needed for certain functions other than αvβ3/αvβ5 mediated migration.

Several recent studies have suggested that the Rho family of small GTPases may be involved in angiogenesis and tumor progression [45–48]. A study by Fernandez-Zapico et al [46] have revealed a novel role of hematopoietic specific GTPase exchange factor, VAV1 in tumorigenesis. Interestingly, Ming et al [49] recently reported that VAV1 (exchange factor for RAC) specifically associates with RAC2 but not RAC1 following fMLP stimulation in human neutrophils. Our studies demonstrate (Fig. 7) that RAC2 is not activated following integrin stimulation when cotransfected along with the mutated SYK (Y342F/Y346F). It has been reported by different laboratories [42,50] that one of the tyrosines (Y342) of the three phosphotyrosine sites in the linker region of SYK is a conserved binding site for VAV. Hence, we argue that the activation of RAC2 could be mediated through SYK potentially via the VAV1 exchange factor. Given the importance of integrins (αvβ3/αvβ5 or a4β1) in the regulation of endothelial cell migration, this interaction between the integrin and SYK may be important for the downstream activation of RAC2-GTP. Consistent with this model the SYK-RAC2 signaling axis is likely to play a pivotal role in the regulation of migration hence control postnatal angiogenesis. Moreover, other laboratories have reported evidence that a link between SYK and Rac1 in T cell signal transduction pathways leading to the activation of JNK kinase [51].

Considerable information has emerged concerning fundamental aspects of angiogenesis from the study of various genetic mouse models. The work of Benezra et al has defined an unexpected role for the Id proteins, Id1 and Id3, in the mouse angiogenic response in vivo [3]. VEGF haploinsufficient mice display a profound defect in angiogenesis as manifested by embryonic lethality on day 11–12 in utero as a result of impaired angiogenesis and hematopoiesis [52].
In contrast, the defect observed in the Rac2−/− mouse is restricted to the adult animal. Similar findings in postnatal angiogenesis were also reported by Mahabeleshwar et al [53]. These studies demonstrated impaired neovascularization in mutant-β3 knock-in adult mice (mice with β3 integrins containing two mutated tyrosine residues, Y747F and Y759F, known to be involved in integrin signaling). Cowan et al reported that the interaction of SYK with β3 integrin is mediated by the intracytoplasmic domain of the β3 integrin subunit which contain NXXY motif position Y747 and Y759 [54]. Importantly, SYK has been reported to bind to the Y747/Y759 β3 subunit via an N terminal tandem SH2 domain interaction. Our results may suggest a molecular basis for the postnatal angiogenic defect observed in the β3 knock-in mouse model, the requirement for the β3 integrin to link to the downstream activation of SYK and RAC2 in ECs. The results of Mahabeleshwar et al support our findings that SYK is required for RAC2 and β3 integrin signaling in endothelial cells. Our data demonstrate that β3 integrin requires RAC2 in endothelial cells for functional integrin signaling and for angiogenesis in vivo. Our results together with the other work cited above suggest a model in which the αvβ3-Syk-Rac2 signaling axis is required for postnatal angiogenesis [53] and is not involved in embryonic angiogenic processes.

Recent evidence suggests that mature vessels e.g. aorta and umbilical vein contain endothelial and endothelial precursor (EPC) populations with significant proliferative potential [55,56]. The source of endothelial cells and EPC in mature vessels and the connection between the hemangioblast, bone marrow-derived and/or blood-derived EPC and the tissue associated EPC is not clear at this time. Our results show that baboon endothelial cells express RAC2 and SYK and they migrate well on the extracellular matrix proteins, fibronectin and vitronectin. Importantly, the conversion of RAC2 to its GTP bound state is augmented when primate ECs are plated on fibronectin as compared to vitronectin and the RAC2 activation event is directly correlated with the magnitude of migration e.g. RAC2 is more robustly activated on FN > VN (Fig. 6). Experiments performed in an aortic ring model suggests that the RAC2 protein functions in the endothelial cells contained within these mature vessels to support elements of angiogenesis including matrix remodeling (MMP9 activity, data not shown) and/or cell migration both components of the endothelial cell outgrowth response (Fig. 3). Additional work is required to elucidate further the molecular mechanism by which RAC2 exerts its pro-angiogenic effects and how SYK orchestrates this response.

In summary, our work demonstrates that RAC2 is expressed at significant levels (comparable to levels of RAC1) in endothelial cells and RAC2 is also a requisite GTPase for endothelial cell migration (via certain integrins, e.g. αvβ3, α4β1, α5β1), the aortic ring endothelial outgrowth response in vitro and neovascularization of the hindlimb following ischemic injury. Moreover, the Rac2−/− animals are markedly deficient in the neovascular response in vivo to bFGF-matrigel induced angiogenesis plug model. Our preliminary results also suggest that RAC2 is involved in neovascularization by mobilizing bone marrow derived endothelial cells to the ischemic region (data not shown). Genetic models and data obtained from heterologous COS7 cell experiment indicate that SYK and RAC2 act in concert to regulate αvβ3 integrin directed migration. Furthermore, from our in vitro and in vivo data related to neovascularization, we suggest that SYK-RAC2 axis (in myeloid/endothelial cells) is necessary for neovascularization. In conclusion, our data provide evidence to establish a novel functional link between “angiogenic integrins” (αvβ3, α4β1 or α5β1), nonreceptor protein tyrosine kinase (SYK) and small GTPase (RAC2) in the regulation of endothelial cell migration and postnatal neovascularization.

Acknowledgments

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BMEC</td>
<td>bone marrow derived endothelial cells</td>
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<td>EC</td>
<td>endothelial cells</td>
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<td>MEF</td>
<td>mouse embryo fibroblasts</td>
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<td>VN</td>
<td>vitronectin</td>
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<td>FN</td>
<td>fibronectin</td>
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<td>LDPI</td>
<td>laser doppler perfusion imaging</td>
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**References**


A

a. Negative control
b. BM Endothelial cells vWF staining
c. BM Macrophage vWF staining
d. BM Endothelial cells ac-LDL uptake

B

BMEC

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Fig. 1. RAC2 expression in endothelial cells
A Presence of vWF and uptake of ac-LDL in bone marrow derived endothelial cells: Expression of endothelial cell marker vWF and uptake of ac-LDL on 6 day cultured bone marrow derived endothelial cells. Cultured EC showed high level of vWF staining (b), and ac-LDL uptake (d). Corresponding negative controls were shown (isotype control) (a), and bone marrow derived macrophages stained with vWF(c). B. Cultured ECs were collected, and total RNA was harvested for examination by RT-PCR using a standard TRIzol extraction procedure performed as written by the manufacturer (Gibco BRL). We evaluated gene products specific for the endothelial cell lineage. Oligonucleotide primer sequences included: CD144-5' : 5'-ggatgcagaggctcacagag-3', CD144-3': 5'-ctggcggttcacgttggact-3', CD144-3' nested: 5'-ctttcacatagtggggcagc-3'; CD31-5': 5'-gctagcggcatgtgagta-3', CD31-3': 5'-ctcctcggcatcttgctgaa-3', CD31-3' nested: 5'-gaagtcgtaggatctccact-3', Flk1-5': 5'-tctgattctcctccttctttaca-3', Flk1-3': 5'-gtatcatttccaaccaccct-3', Flk1-3' nested: 5'-gtgttttgcagaagatactg-3'. The PCR products were visualized with ethidium bromide in 2% agarose gels. β-Actin was used as a loading control. C. Expression of GFP positive tissues from brain (a), lung (b), kidney (c) and heart (d) within the endothelium of Tie2 transgenic adult mice. D. Hematopoietic and endothelial cell specific gene profile of adult bone marrow and adult heart derived from the Tie2 GFP mouse: RT-PCR analysis showed the gene expression profile of cells from adult bone marrow and cells sorted cells from Tie2 GFP positive cells present within adult heart tissue. The bone marrow Sca-1+/c-kit +/lin- cells express hematopoietic specific genes [embryonic hemoglobin (βH1), adult hemoglobin (β Major) Gata 1 etc] as well as CD31 whereas Tie2 GFP positive cells from adult heart express endothelial specific gene transcripts [von Willebrand factor (vWF), Tie2, CD31, VE cadherin (CD144) etc]. These data are representative of three independent experiments performed. E. To determine whether RAC2 was expressed in endothelial cells from different experimental sources, we performed immunoblot analysis using anti-RAC2 specific antibody. Whole cells lysates were prepared from human brain endothelial cells (HBEC), human umbilical vein endothelial cells (HUVEC), EGFP sorted (green) heart endothelial cells from Tie2 transgenic mice. Bone marrow derived endothelial cells from Rac2 +/+ or Rac2 −/− mice were compared for RAC2 expression. As positive control, mouse macrophage cell line (J774A.1), bone marrow
derived macrophages from Rac2 wild type mice and as negative control, bone marrow derived macrophages from Rac2 knockout mice and mouse embryo fibroblast derived from Rac2 +/- animal were used. A β-actin Western blot was used as a loading control.
Fig. 2. RAC2 controls vitronectin (VN), H296 (α4β1) and CH271 (α5β1) migration in bone marrow derived endothelial cells

A. Bone marrow derived endothelial cells from Rac2 +/+ (WT) and −/− (KO) mice were tested in haptotaxis assay for capacity to migrate on vitronectin or fragments of fibronectin corresponding to specific integrin directed migration. VN, vitronectin; H296, via α4β1; CH271 via α5β1. A comparison between WT and KO cells on VN shows significant difference (p<0.005), comparison of WT to KO cells for migration on the H296 peptide shows a significant difference (p<0.001), and on CH271 peptide (p<0.05). Data represent mean ±SD, representative of 5 experiments performed.

B. Western blot analyses were employed to determine the levels of expression of RAC2 and RAC1 in bone marrow derived EC evaluated in panel A. ECs were derived from WT and KO mice and mouse embryo fibroblast were from Rac2 +/+ animals. Whole cell lysates were prepared from Rac2 +/+ or −/− bone marrow derived endothelial cells or MEF and probed with RAC2 specific rabbit anti-sera or monoclonal antibody of RAC1. A β-actin Western blot was used as a loading control. Data are representative of five experiments performed.

C. Mature bone marrow derived endothelial cells were transiently transfected with mock siRNA, control siRNA (non specific control pool with low GC content), three different non overlapping sequences of Rac2 siRNA (from Invitrogen, Stealth siRNA, 1, Rac2 –MSS237710, 2, Rac2-MSS237711 and 3, Rac2-MSS237712) or a combination of 1, 2 and 3 at a concentration of 20 nM by Lipofectamine 2000 reagent. The cells were harvested and analyzed after 24 and 48 hours of transfection. i) cell lysates were subjected to immunoblotting with anti RAC2 (upper panel) and β actin antibodies (lower panel). Blot shows that all three different sequences of siRNA and pool of siRNA (combination of 1, 2 and 3) significantly inhibit RAC2 protein expression. ii) migration on vitronectin (αvβ3/αvβ5) of Rac2 siRNA transfected (24 and 48 hours) wild type bone marrow derived endothelial cells were significantly less compare to control (mock and low GC control group).
Data represent mean + SD (n = 4, p<0.005 at 24 hours and p<0.001 at 48 hours). D. Bone marrow derived endothelial cells were transiently transfected with mock siRNA, control siRNA (non-specific control pool), three different non-overlapping sequences of Rac1 siRNA (from Invitrogen, Stealth siRNA, 1, Rac1 –MSS237707, 2, Rac1-MSS237708 and 3, Rac1-MSS237709), or a combination of 1, 2 and 3 at a concentration of 20 nM by Lipofectamine 2000 reagent. The cells were harvested and analyzed after 48 hours. i) cell lysates were subjected to immunoblotting with anti RAC1 (upper panel) and β actin antibodies (lower panel). Blot shows that all three different sequences of siRNA and pool of siRNA (combination of 1, 2 and 3) block RAC1 protein expression. ii) migration on vitronectin (avβ3/avβ5) of Rac1 siRNA transfected (24 and 48 hours) wild type bone marrow derived endothelial cells were unchanged (n = 4 p<0.2 comparison of all groups). From these data and Rac2 knockout, data we can conclude that integrin directed bone marrow derived endothelial cell migration is dependent on RAC2-GTPase but not on RAC1-GTPase.
Aortic ring endothelial outgrowth response in Rac2−/− mice

A The aortic ring outgrowth response was compared in matrigel embedded aortic ring explants isolated from Rac2+/+ versus Rac2−/− mice. Aortic rings were cultured for 7 days at 37°C (n=5 per group per experiment). The results shown are representative of 5 aortic rings per experimental group evaluated and 3 separate experiments performed. B A quantitation of the
aortic ring outgrowth assay comparing the Rac2 $-/-$ to $+/+$ mice. Number of aortic rings evaluated per mouse equals 5; number of mice per experimental group was 7. * $p < .0001$ comparing $-/-$ to $+/+$ animals.
Fig. 4. RAC2 is required for neovascularization of the hindlimb following ischemic injury

A. Examination of hindlimb perfusion by Laser Doppler Perfusion Imaging (LDPI) disclosed profound defect in limb reperfusion following ligation of the femoral artery in Rac2 −/− animals. In contrast, significant improvement in the limb perfusion was noted within 7 days in Rac2 +/+ mice (n = 7 per group per experiment). These are the representative Laser Doppler Images taken on day 7 of the experiment.

B. Quantitative analysis of perfusion recovery, measured by LDPI was compared in Rac2 −/− versus +/+ mice. From day 4 onwards postoperatively limb perfusion was severely reduced in Rac2 −/− mice (P<0.04).
subsequent 21 days, limb perfusion remained markedly depressed in Rac2 deficient mice compared to wild type mice.
Fig. 5. RAC2 is essential for FGF-induced angiogenesis in an in vivo matrigel angiogenesis assay
The upper panel shows a representative anti-VEGFR2 (FLK-1) Western blot performed on matrigel plug lysates derived from Rac2 −/− versus Rac2 +/+ mice. Lower panels show CD31 staining for microvessels within matrigel plugs 5 days after implantation into Rac2 −/− versus +/+ mice. n = 6 animals per group studied.
Fig. 6. Primate endothelial cells activate RAC2 upon integrin engagement

A We quantitated RAC2 and RAC1 activation following adhesion to vitronectin (VN) or fibronectin (FN) for 15 and 30 minutes. NS, no stimulation. Conversions of GDP-RAC2 to GTP-RAC2 or GDP-RAC1 to GTP-RAC1 were measured by adding GST fusion protein representing the GTP-RAC2 or GTP-RAC1 binding CRIB domain of the PAK1 kinase. All cell lysates contained equal amount of total protein per sample. Densitometric measurements of assays shown in Fig. A following the Western blots of RAC2-GTP and RAC1-GTP respectively. **B.** Primate endothelial cells were tested in a haptotaxis assay for capacity to migrate on two different matrix proteins (vitronectin and fibronectin). The data are representative of three individual experiments performed.

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PULLDOWN WITH PAK-PBD

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Immunoblot

B

- Rac2+pEGFP
- Rac2+WT-Syk
- Rac2+KD-Syk
- Rac2+Y317F
- Rac2+Y342/Y346F
- Rac2+Y317/Y342/Y346F

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Fig. 7. Enzymatic activity and linker region of SYK are essential for integrin directed migration and RAC2 activity

COS7 cells were transiently transfected with episomal plasmids coding for RAC2 and wild type SYK-EGFP or SYK-EGFP containing indicated point mutations (catalytically dead SYK, K396R-KD-SYK; SYK-Y317F; SYK-Y342F/Y346F and Y317F/Y342F/Y346F). **A.** RAC2 activity was determined using GST-CRIB-PBD1 pull down assay (see details in Materials Methods) following ligand (H296, fragmented peptide of fibronectin) stimulation for 15 minutes (NS: non stimulated). Cell lysates were used for RAC2 activation assays after protein equilibration. **B.** Photographs of lower aspect of transwell membrane, a measure of integrin (a4β1, for H296) directed migration of COS7 cells transfected with different mutants of SYK (see above). **C.** Integrin directed migration of COS7 cells transfected with RAC2 or different mutant forms of SYK (see above). Following transfection migration response on H296 peptide was quantified as described under “Materials and Methods”. Bars represent S.D of the mean. **D.** Western blot analysis of RAC2 and total RAC expression in COS7 cells. All cell lysates contained equal amount of total protein per sample.
Fig. 8. SYK-RAC2 signaling axis is required for αvβ3/αvβ5 migration
A BMECs from WT, Rac2−/+), Rac2−/−, Syk−/− and Syk−/+:Rac2−/+ mice were tested in a migration assay for capacity to migrate on VN. One-way analysis of variance (ANOVA) was

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used to test the equality of means for the groups: WT, RAC2−/+; RAC2−/−, SYK−/+; SYK−/+
−;RAC2−/+. A posteriori Tukey’s method with a family error rate of 0.05 was used for the
pairwise comparison between group means. Tukey 95% simultaneous confidence intervals
from all pairwise comparisons among five groups indicated there were significant difference
between any two groups, except RAC2−/+ vs. SYK−/+, and RAC2−/− vs. SYK−/++;RAC2−/+ (p<0.05 was considered significant).

Bone marrow derived endothelial cells from WT, Rac2−/+; Rac2−/−; Syk−/+; Syk−/++;Rac2 −/+ (double heterozygote) mice were tested in haptotaxis assay for capacity to migrate on vitronectin (avβ3/avβ5). B. A recombinant PAK-CRIB domain binding assay (pull-down assay) was performed to determine the level of RAC2-GTP or RAC1-GTP in cell lysates of WT (lane 2), Rac2−/+ (lane 3), Syk−/+ (lane 4) and Syk−/++;Rac2−/+ (lane 5). Cell lysates were used for RAC2 and RAC1 pull-down assay after protein equilibration. Lane 6 is the
positive control (lysates was treated with 10 mM EDTA and 100μM GTPγS at 30°C for 15
minutes before addition of PAK1-PBD glutathione agarose conjugate). NS, no stimulation.

Cell lysates used in this Western blot contained equal amount of protein per lane. Total RAC2
and RAC1 were immunoblotted for loading control. Data (A and B, upper panel) show that
both integrin directed migration and activation of RAC2 (but not RAC1) are significantly less
in Rac2−/+ as well as Syk−/+ bone marrow endothelial cells when compare to WT cells.

Integrin directed migration and activation of RAC2 are further decreased in Syk−/++;Rac2−/+ cells.