Receptor-mediated signaling is required for endothelial cell proliferation and migration, processes that are critical to blood vessel formation and function.\textsuperscript{1} Signaling downstream of receptor engagement in endothelial cells leads to the activation of several pathways, including phospholipase C-\(\gamma\) activation, to produce diacylglycerol (DAG). DAG in turn activates downstream targets to affect endothelial cell behaviors. Signals that are upregulated in diabetes, such as vascular endothelial growth factor-A (VEGF-A) and endothelin-1 (ET1), generate DAG. DAG is also produced by aberrant glucose metabolism, and DAG levels are elevated in diabetic animals and patients.\textsuperscript{2,3} Diabetic individuals have compromised angiogenesis and blood vessel function, and fetuses of diabetic mothers have an increased incidence of birth defects, including vascular defects.\textsuperscript{3,4} It is assumed that these vascular defects are mediated, at least in part, by elevated DAG levels, because mouse embryos recovered from diabetic mothers had elevated DAG levels and increased developmental defects;\textsuperscript{3} however, it is not fully understood how elevated DAG leads to vessel dysfunction.

The effects of DAG on cell signaling are mimicked by phorbol esters, tumor promoters that also affect endothelial proliferation, cellular morphology, apoptosis, and barrier function.\textsuperscript{5-9} DAG and phorbol esters are potent activators of the protein kinase C (PKC) family of proteins. Numerous mammalian PKC isoforms fall into several subfamilies, and DAG/

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**Rationale:** Fetuses that develop in diabetic mothers have a higher incidence of birth defects that include cardiovascular defects, but the signaling pathways that mediate these developmental effects are poorly understood. It is reasonable to hypothesize that diabetic maternal effects are mediated by 1 or more pathways activated downstream of aberrant glucose metabolism, because poorly controlled maternal glucose levels correlate with the frequency and severity of the defects.

**Objective:** We investigated whether RasGRP3 (Ras guanyl-releasing protein 3), a Ras activator expressed in developing blood vessels, mediates diabetes-induced vascular developmental defects. RasGRP3 is activated by diacylglycerol, and diacylglycerol is overproduced by aberrant glucose metabolism in diabetic individuals. We also investigated the effects of overactivation and loss of function for RasGRP3 in primary endothelial cells and developing vessels.

**Methods and Results:** Analysis of mouse embryos from diabetic mothers showed that diabetes-induced developmental defects were dramatically attenuated in embryos that lacked Rasgrp3 function. Endothelial cells that expressed activated RasGRP3 had elevated Ras-ERK signaling and perturbed migration, whereas endothelial cells that lacked Rasgrp3 function had attenuated Ras-ERK signaling and did not migrate in response to endothelin-1. Developing blood vessels exhibited endothelin-stimulated vessel dysmorphogenesis that required Rasgrp3 function.

**Conclusions:** These findings provide the first evidence that RasGRP3 contributes to developmental defects found in embryos that develop in a diabetic environment. The results also elucidate RasGRP3-mediated signaling in endothelial cells and identify endothelin-1 as an upstream input and Ras/MEK/ERK as a downstream effector pathway. RasGRP3 may be a novel therapeutic target for the fetal complications of diabetes. (Circ Res. 2011;108:1199-1208.)

**Key Words:** blood vessel disruption ■ diabetic embryopathy ■ Ras activator ■ endothelial migration ■ actin cytoskeleton ■ endothelin-1
phorbol esters activate PKC signaling by binding to C1 domains found in the classic (α, β, γ) and novel (δ, ε, η, θ) subgroups of PKCs. Recently, several non-PKC protein families were identified that contain C1 domains and respond to DAG and phorbol esters, which suggests that some DAG/phorbol ester-mediated responses require these proteins.

Ras guanyl-releasing proteins (RasGRPs) are non-PKC DAG/phorbol ester receptors that function as guanine nucleotide exchange factors and activate the Ras family of GTPases. Ras proteins are localized to membranes, and their activation is controlled in part by proximity to guanine nucleotide exchange factors, such that recruitment of guanine nucleotide exchange factors to membranes can activate Ras. Both DAG and phorbol esters recruit RasGRPs to membranes via the C1 domain. Active Ras in turn activates several effector pathways such as MEK/ERK, p38 mitogen-activated protein kinase (p38 MAPK), and JNK. Ras proteins are activated by phorbol esters in cultured endothelial cells, and PKCs also contribute to Ras and ERK activation in response to phorbol esters in endothelial cells; however, the potential role of non-PKC DAG/phorbol ester receptors in this response has not been investigated.

The RasGRP family of non-PKC phorbol ester receptors has 4 family members. The RasGRPs have limited sites of expression in vivo, and genetic deletion experiments reveal a nonredundant function for the RasGRPs in hematopoietic and endothelial cells. RasGRP1 transduces signals downstream of the T-cell receptor for T-cell maturation, and both RasGRP1 and RasGRP3 affect B-cell function in complex ways. RasGRP2 recently was reported to have effects on vascular development in Xenopus. We identified RasGRP3 in a murine-based gene-trap screen as a locus expressed in endothelial cells. RasGRP3 transduces signals downstream of DAG that affect endothelial cell behaviors and lead to vessel dysmorphogenesis. Thus, excess DAG in diabetes likely overactivates RasGRP3, and this contributes to the perturbed development of fetuses in diabetic environments.

**Methods**

**Diabetic Mice and Embryo Analysis**
Female mice (C57Bl/6J purchased from The Jackson Laboratory, Bar Harbor, ME, or Rasgrp3<sup>−/−</sup> (a loss-of-function null mutation<sup>19</sup> backcrossed to N8 on the C57Bl/6J background) at 6 to 8 weeks of age were made diabetic according to the protocol in the Animal Models of Diabetic Complications Consortium. Alternatively, Ins<sup>2</sup>Akita<sup>−/−</sup> mice on the C57Bl/6J background (The Jackson Laboratory, #003548) were bred to obtain Ins<sup>2</sup>Akita<sup>−/−</sup>Rasgrp3<sup>−/−</sup> mice. Blood glucose was monitored weekly, and mice with blood glucose levels >250 mg/dL were considered diabetic. Mice were mated to genotype-matched males, and embryos were harvested at embryonic day 9.5 (E9.5), fixed, and whole-mount stained for platelet endothelial cell adhesion molecule as described previously. Stained embryos were imaged and scored for defects as described in the Online Data Supplement (available at http://circres.ahajournals.org).

Whole-embryo culture was performed on embryos dissected at E7.5 or E8.5, in roller bottles as described previously. Some embryos were treated with PMA (phorbol 12-myristate 13-acetate) 50 nmol/L and some with glucose 20 mmol/L during the culture period. After 24 hours, embryos were removed and photographed.

**Endothelial Cells**
Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA), cultured according to the manufacturer’s protocol, and used between passages 2 and 8. Transfections were performed with an Amaxa Nucleofector (Lonza, Basel, Switzerland) according to directions. For staining, cells were fixed, permeabilized, blocked, and incubated with Alexa Fluor 555-phalloidin (Molecular Probes, Carlsbad, CA). Migration assays were performed 48 hours after transfection as described in the supplemental Methods. Average velocity and distance to origin of cells were calculated with trajectory measures as diagrammed in Figure 3J.

Mouse endothelial cells (wild type and Rasgrp3<sup>−/−</sup>) were generated and expanded as described previously, with minor modifications. Proliferation assays and migration assays were as described in the Online Data Supplement.

**Embryonic Stem Cell Differentiation and Analysis**
Wild-type (WT; <sup>27</sup>) and Rasgrp3<sup>−/−</sup> embryonic stem (ES) cells were maintained and differentiated for 8 days as described previously. For inhibitor studies, day 7 ES cell cultures
fore, we examined the effects of elevated DAG/phorbol ester, and the diabetic environment on these stages of mouse development. We first mimicked elevated DAG signaling by glucose, and the diabetic environment on these stages of mouse development. We first mimicked elevated DAG signaling by addition of PMA 100 nmol/L for 24 hours. For ET1 stimulation, ET1 100 nmol/L in fresh medium was added daily between days 5 and 8. Antibody staining of ES cell cultures was as described previously.25 Quantitative image analysis of platelet endothelial cell adhesion molecule–stained ES cell cultures was performed as described previously.25

Ras and ERK Activation Assays

HUVECs (50% to 80% transfected by green fluorescent protein labeling after transfection) or mouse endothelial cells were grown to near confluence for 48 hours, serum-starved overnight, then processed as described in the supplemental Methods for Ras-GTP immunoprecipitation or total Ras, phosphorylated ERK, or total ERK Western blots.

Results

Loss of Rasgrp3 Protects Embryos From Diabetes-Induced Birth Defects

Because RasGRP3 is expressed in developing vessels and somites of midgestation mouse embryos,19 we reasoned that embryos exposed to elevated DAG signaling would be susceptible to effects of RasGRP3-mediated signaling in vivo. Therefore, we examined the effects of elevated DAG/phorbol ester,
tive to Rasgrp3<sup>−/−</sup> mutant embryos (Online Figure III). To make more rigorous comparisons, we next examined embryos on the C57Bl6/J inbred background. We induced diabetes chemically via streptozotocin (STZ), and we also used mice carrying the Ins2<sup>Akita</sup> mutation that induces diabetes genetically. We monitored the average maternal blood glucose at the time of euthanasia via tail bleed and found the following values: WT STZ, 381 mg/dL; Rasgrp3<sup>−/−</sup> STZ, 448 mg/dL; Ins2<sup>Akita</sup>; Rasgrp3<sup>−/−</sup>, 387 mg/dL. Embryos were harvested from diabetic mothers at E9.5 and whole-mount stained for platelet endothelial cell adhesion molecule to visualize vessel development and patterning, and the severity of defects in head plexus vessels, intersomitic vessels, and trunk/somite was scored as described in Methods (Figure 1). WT embryos from mothers with diabetes that was induced either chemically or genetically had a significantly elevated developmental severity index compared with controls (Figures 1A, 1B, 1E, and 1O; Online Figure IV). Closer examination of the somite region showed defects in somites and in the pattern of intersomitic vessels (Figures 1G through 1J), and similar defects were seen in the vascular plexus of the head (Figures 1 K through 1N). In contrast, embryos similarly developing in diabetic mothers but genetically deficient for Rasgrp3 appeared relatively unaffected, and their developmental severity index was significantly lower than WT counterparts and close to control levels (Figures 1C through 1O; Online Figure IV). Analysis of yolk sacs from these embryos showed a low level of vascular defects that did not significantly correlate with genotype, diabetic condition, or the severity of the corresponding embryo (Online Figure V and data not shown). Thus, loss of Rasgrp3 function had a protective effect on diabetes-induced birth defects.

Ras Is a Target of RasGRP3 in Endothelial Cells
To explore how RasGRP3 signaling contributes to diabetes-induced developmental defects, we first analyzed signaling in HUVECs that upregulated RasGRP3 activity, because the developmental defects are predicted to result from overactivation of RasGRP3 by DAG (Figure 2). WT RasGRP3 linked to a green fluorescent protein reporter and constructs in which a K-Ras CAAX or H-Ras CAAX sequence were transiently expressed in HUVECs (Figure 2A). The CAAX sequences localize RasGRP3 to membranes, which mimics DAG-promoted activation to place the activator in physical proximity to Ras. Thus, these constructs are predicted to be “constitutively active” in terms of their action on Ras and provide gain-of-function activity independent of any overexpression effects. Overexpression of WT RasGRP3 did not significantly increase Ras activation over baseline, but both of the CAAX-tagged RasGRP3 proteins stimulated significant Ras activation (Figures 2B and 2C). ERK activation is downstream of Ras activation, and in HUVECs, both CAAX-tagged RasGRP3 proteins significantly induced ERK activation (Figures 2B and 2C). These data indicate that Ras is a target of RasGRP3 in endothelial cells.

To examine the cellular responses of endothelial cells to RasGRP3-mediated signaling, we examined the effects of RasGRP3 overactivation on the cytoskeleton, because in other cell types, Ras activation affects the cytoskeleton. Analysis of yolk sacs from these embryos showed a low level of vascular defects that did not significantly correlate with genotype, diabetic condition, or the severity of the corresponding embryo (Online Figure V and data not shown). Thus, loss of Rasgrp3 function had a protective effect on diabetes-induced birth defects.
The microtubule staining pattern was not affected by overexpression of RasGRP3 (data not shown); however, the actin cytoskeleton was dramatically altered in endothelial cells that overexpressed membrane-localized RasGRP3 (Figures 3E through 3H). RasGRP3-CAAX–expressing cells appeared larger and flatter, and they had very few actin stress fibers. Cortical actin was also diminished in endothelial cells that overexpressed RasGRP3-CAAX. Because actin stress fibers are implicated in proper migration, we investigated the migratory behavior of endothelial cells that overexpressed RasGRP3-CAAX. Because actin stress fibers are implicated in proper migration, we investigated the migratory behavior of endothelial cells that overexpressed RasGRP3 (Figures 3I through 3L). Endothelial cells that overexpressed RasGRP3 were identified by green fluorescent protein expression, and all cells were labeled with cell tracker (Figure 3I). Cells were imaged, and the average velocity and distance migrated from origin were calculated as described in Methods (Figure 3J). None of the RasGRP3 proteins affected the overall velocity of the cells, but the CAAX-tagged RasGRP3 proteins both significantly inhibited the distance migrated from the origin (Figures 3K and 3L). These results show that endothelial cells that overexpress membrane-localized (and thus activated) RasGRP3 have a perturbed actin cytoskeleton and an attenuated ability to migrate in a forward direction.

We investigated whether RasGRP3 was necessary for Ras and ERK activation downstream of DAG/phorbol ester stimulation. WT and Rasgrp3gt/gt endothelial cells incubated with PMA were evaluated for changes in the levels of Ras and ERK activation. WT endothelial cells stimulated with PMA had elevated levels of active Ras and active ERK. In contrast, Rasgrp3gt/gt endothelial cells stimulated with PMA showed no detectable Ras activation, and ERK activation was attenuated (Figure 4A; Online Figure X, A). These results reveal a requirement for RasGRP3 in Ras/ERK signaling downstream of DAG/phorbol ester stimulation in endothelial cells.

We next examined the signaling requirements downstream of activated RasGRP3 in developing vessels using a mouse ES cell–differentiation model that supports the formation of primitive blood vessels in vitro via a programmed differentiation.39,32,33 We previously showed that ES cell–derived blood vessels respond to phorbol ester stimulation with a dramatic vessel dysmorphogenesis that is dependent on Rasgrp3 function19 (Online Figure VII, A–D). We therefore investigated whether signaling downstream of activated RasGRP3 in developing vessels used the Ras effector pathways MEK/ERK, p38 MAPK, or JNK. The vessel dysmorphogenesis seen on PMA stimulation was

Figure 3. Overexpression of active RasGRP3 perturbs the actin cytoskeleton and migration of endothelial cells. A–H, HUVECs transfected with the indicated constructs were incubated for 48 hours, then fixed and imaged for green fluorescent protein (GFP, green; A–D) or stained with phalloidin (red; B–H). A and B, Control GFP-CAAX; C and D, GFP-RasGRP3; E and F, GFP-RasGRP3-KCAAX; G and H, GFP-RasGRP3-HCAAX. In E–H, * indicates transfected cells with perturbed phalloidin staining. I and J, HUVECs were labeled with cell tracker and live-imaged. I, HUVECs expressing GFP-RasGRP3 (green) and labeled with cell tracker (red). J, Diagram showing how the average velocity (V) and distance from origin (D) were calculated from cell trajectories. K, Average velocity. L, Average distance from origin. **P ≤ 0.05 and ***P ≤ 0.001 relative to control.
RasGRP3 Mediates Vessel Dysmorphogenesis Induced by ET1

Because both ET1-induced endothelial cell proliferation and migration are RasGRP3-dependent, we investigated whether ES cell–derived blood vessels had RasGRP3-dependent dysmorphogenesis induced by ET1 (Figure 6). ET1 treatment of WT vessels led to loss of the fine vascular network and significantly increased vascular area (Figures 6A, 6B, and 6E). This response was Rasgrp3gt/gt dependent, because Rasgrp3m/m endothelial cells retained the fine vascular network and did not exhibit increased vascular area with ET1 treatment (Figures 6A, 6B, and 6E). ET1-mediated vessel dysmorphogenesis was also dependent on PKC activity, because PKC inhibition blocked ET1 effects on vessels (Online Figure IX). Thus, developing vessels exhibited vessel dysmorphogenesis in response to ET1, and that response required RasGRP3 and PKC function. Taken together, these data suggest

RasGRP3 Is Required for ET1-Mediated Ras and ERK Signaling in Endothelial Cells

Although phorbol ester mimics endogenous DAG production and signaling downstream of physiological inputs, it does not identify the physiological signals that normally activate RasGRP3-dependent signaling in endothelial cells. To identify physiologically relevant signals upstream of RasGRP3 in endothelial cells, we investigated signaling mediated by VEGF-A and ET1, 2 angiogenic factors that use DAG-Ras-ERK downstream signaling. WT and Rasgrp3gt/gt endothelial cells were stimulated with VEGF or ET1, and levels of active Ras and ERK were analyzed (Figures 4B and 4C; Online Figure X, B). As predicted, WT endothelial cells had increased levels of active Ras and ERK on treatment with either VEGF or ET1. In contrast, although Rasgrp3gt/gt endothelial cells had elevated levels of active Ras and ERK with VEGF treatment, they showed no increase in active Ras and attenuated active ERK with ET1 stimulation. Thus, RasGRP3 is required for Ras and ERK activation downstream of ET1 stimulation in endothelial cells. These results identify ET1 as a physiological signal for RasGRP3-mediated signaling in endothelial cells.

Figure 4. RasGRP3 is required for Ras-ERK activation downstream of phorbol ester and ET1 but not VEGF-A. A, WT and Rasgrp3gt/gt endothelial cells were treated with PMA and processed for Ras and ERK activation. Compared with WT endothelial cells, Rasgrp3gt/gt endothelial cells did not show increased activated Ras (Ras-GTP) and had an attenuated activation of phosphorylated ERK (pERK). B, WT and Rasgrp3gt/gt endothelial cells were treated with ET1 or VEGF-A and processed for Ras activation. WT endothelial cells had increased levels of activated Ras (Ras-GTP) in response to both VEGF and ET1, whereas Rasgrp3gt/gt endothelial cells had increased activated Ras (Ras-GTP) in response to VEGF-A but an attenuated response to ET1. C, WT and Rasgrp3gt/gt endothelial cells were stimulated with ET1 or VEGF-A and processed for ERK activation. WT endothelial cells had increased levels of pERK in response to both VEGF and ET1, whereas Rasgrp3gt/gt endothelial cells had increased pERK in response to VEGF-A but attenuated in response to ET1. Experiments are representative of at least 3 replicates.

significantly attenuated in the presence of the MEK inhibitor U0126 (Online Figure VII, E–F, I). In contrast, the p38 MAPK inhibitor SB203580 had no effect on PMA-induced vessel dysmorphogenesis (Online Figure VII, G–I), and the JNK inhibitor SP600125 showed a similar lack of effect (data not shown). Thus, Rasgrp3-dependent DAG/phorbol ester signaling in developing vessels requires MEK but not p38 MAPK or JNK downstream of Ras GTases.

Activation of RasGRP3 in B cells requires, in addition to membrane localization, phosphorylation by PKC. To test PKC function in endothelial cells, we exposed ES-derived vessels to PKC inhibitors concomitant with exposure to phorbol ester (Online Figure VIII). A general inhibitor of most PKC isoforms, BIM (bisindolylmaleimide), targets the kinase activity of PKC and not C1 domain interactions; thus, BIM inhibits PKC activation without affecting C1-domain–mediated RasGRP3 activation. BIM completely blocked phorbol ester–induced vessel dysmorphogenesis (Online Figure VIII, C–D, I). Likewise, an inhibitor of the conventional PKC isoforms α and β, Gö6976, completely blocked PMA-induced vessel dysmorphogenesis (Online Figure VIII, E–F, I). A third PKC inhibitor, rottlerin, predominantly affects PKC-δ, and it partially blocked PMA-induced vessel dysmorphogenesis (Online Figure VIII, G–I). These results show a requirement for PKC activity in phorbol ester–induced vessel dysmorphogenesis and suggest that endothelial RasGRP3 requires activation by PKC phosphorylation.
that RasGRP3 is required for ET1-induced endothelial cell angiogenic responses.

Discussion

RasGRP3 is an activator of Ras family GTPases that is expressed in angiogenic vessels and is required for the DAG/phorbol ester–mediated responses of these vessels. Here, we posited that RasGRP3 is overactivated in embryos that develop in a diabetic environment and are susceptible to birth defects, and we showed that loss of RasGRP3 significantly attenuated the detrimental effects of a diabetic environment on embryonic vascular development. We also defined the molecular and cellular processes perturbed by DAG/phorbol ester–induced activation of RasGRP3 activation in endothelial cells of developing vessels. We defined ET1 as an upstream input and Ras as a target of RasGRP3 in endothelial cells, and we showed that manipulation of RasGRP3 perturbs endothelial migration. These data led to a model of RasGRP3-mediated signaling in endothelial cells that includes a molecular mechanism, cellular phenotype, and effects on the developing embryo in a diabetic environment (Figure 7).

We activated RasGRP3 in developing vessels using phorbol ester as a DAG mimic, and RasGRP3-dependent vessel dysmorphogenesis required MEK/ERK signaling. The present data indicate that Ras is a primary target of RasGRP3 in endothelial cells, because overexpression of RasGRP3 linked to either K-Ras CAAX or H-Ras CAAX domains significantly activated both Ras and ERK, whereas loss-of-function analysis showed that RasGRP3 was required for ET1- or DAG/phorbol ester–mediated Ras and MEK/ERK activation. The present data do not rule out that RasGRP3 activates other Ras GTPases in endothelial cells, but they suggest that signaling through Ras to MEK/ERK is critical for the vascular response to DAG. RasGRP3 is normally activated by...
both localization to membranes via DAG binding to its C1 domain and PKC phosphorylation. Overexpression of Ras-GRP3 alone was not sufficient to activate endothelial Ras, but overexpression of membrane-localized forms was sufficient for activation, which suggests that endogenous PKC levels are not rate-limiting for RasGRP3 activation in endothelial cells. The present studies also revealed a requirement for classic/novel PKC isoform activity in the response of developing vessels to DAG/phorbol esters and ET1. Although it is possible that the PKC requirement is downstream of the genetic requirement for RasGRP3, we favor the hypothesis that PKC phosphorylation of RasGRP3 is required for its activity in endothelial cells, as has been shown in B cells.

Elevated RasGRP3 activity affects specific cellular responses of endothelial cells. Endothelial cells that overexpress activated RasGRP3 are flattened relative to controls, a phenotype also observed in neural cells that express activated RasGRP3. Consistent with this phenotype, the actin cytoskeleton is perturbed in these cells, with loss of stress fibers and reduction of the cortical actin ring. The perturbation of the actin cytoskeleton in endothelial cells that overexpress RasGRP3 is similar to actin perturbations seen in tumor cells that express activated Ras. In general, Ras activation reduces actin stress fiber formation, primarily through effects on RhoA activity but also via MEK signaling to ERK1/2 and ERK5. Actin stress fibers link to focal adhesions on the ventral side of cells, and this linkage promotes actomyosin contractility. In terms of migration, stress fibers are thought to act as rudders that keep cells migrating directionally. Consistent with this idea, we find that endothelial cells that overexpress activated RasGRP3 have normal velocity but significantly reduced migration relative to the origin over time. This finding suggests that the cells can move, but their ability to link one movement to the next in a coordinated fashion is impaired. Thus, overexpression of activated RasGRP3 mimics Ras overactivation and prevents orderly forward migration of endothelial cells.

Complementary loss-of-function analysis showed that Rasgrp3 function is required for DAG/phorbol ester–mediated Ras activation, as is predicted to occur in diabetic environments with elevated DAG levels. Interestingly, RasGRP3 is involved in Ras/ERK signaling downstream of ET1 in endothelial cells. ET1 is elevated in diabetic milieu and leads to DAG production, so perhaps 2 sources of DAG (DAG produced via elevated glucose and DAG produced via elevated ET1 signaling) contribute to RasGRP3-mediated vascular pathologies in diabetic animals. In contrast, a second signal that is elevated in diabetic environments, VEGF-A, does not appear to require RasGRP3 for Ras-mediated signaling, although RasGRP3 expression is upregulated by VEGF-A. Several studies reported that ET1 angiogenic effects required VEGF-A. In light of our work, it is possible that this VEGF-A requirement reflects a need for VEGF-A–stimulated RasGRP3 expression. Thus, the requirement for RasGRP3 in ET1-mediated signaling may link the activities of the 2 pathways in diabetes.

Because diabetes is accompanied by elevated DAG levels and leads to developmental defects, including vascular defects, we hypothesized that in a diabetic fetal environment, elevated maternal glucose crosses the placenta, where it is metabolized to DAG. The excess DAG ectopically activates embryonic RasGRP3, and downstream activation of Ras signaling contributes to the increased incidence of developmental problems. This hypothesis predicts that embryos that lack Rasgrp3 will be less susceptible to diabetes-induced birth defects, and in fact, in the present study, embryos that lacked Rasgrp3 were significantly protected from developmental defects produced in a diabetic fetal environment. This was true whether the mothers were diabetic as a result of STZ destruction of pancreatic β-cells or via the Ins2Akita mutation, which inactivates the insulin II gene and thus leads to diabetes. The embryonic defects we documented were similar to the defects reported by others. Interestingly, embryos that lacked Rasgrp3 function and developed in diabetic mothers had significantly fewer defects in develop-
ing somites and developing vessels, 2 embryonic organs that express RasGRP3. The vascular defects seen in WT embryos from diabetic mothers were consistent with the endothelial migration defects observed in primary endothelial cells, because the intersomitic vessels were sometimes blunted, with expanded migratory fronts. Interestingly, although yolk sac vessels showed some pattern defects, these did not correlate significantly with embryo genotype, diabetic environment, or overall embryo defects, in contrast to another group who described significant diabetes-induced yolk sac vasculopathy. This indicates that the observed diabetes-induced embryonic defects were not secondary to yolk sac defects that compromised overall embryonic health.

Was the protection afforded by loss of RasGRP3 from the maternal compartment, the embryonic compartment, or both? Several lines of evidence support a critical role for embryonic Rasgrp3 function in mediating the effects of diabetes. First, another study showed that DAG levels and activated PKC are elevated in embryos from diabetic mothers, which indicates that upstream requirements for RasGRP3 activation are in place in the embryo. In the present study, mothers that lacked RasGRP3 became diabetic, and their average blood glucose at the time of euthanasia was higher than WT controls. Whole-embryo culture with medium supplemented with PMA showed that embryos that lacked Rasgrp3 were significantly protected from the severe PMA-induced perturbations seen in WT embryos. Finally, embryos exposed to elevated glucose had increased defects that were RasGRP3-dependent, which shows that the RasGRP3 status of the embryo is critical to its response to maternal glucose. Taken together, these findings indicate that lack of Rasgrp3 function in the embryo is critical for protection from developmental defects, and that potential maternal effects of Rasgrp3 loss do not impact the elevated glucose levels that lead to elevated DAG and diabetes.

The finding that RasGRP3 mediates the effects of a diabetic environment on embryonic development, including vascular development, suggests that RasGRP3 may be a new and useful therapeutic target for prevention of diabetes-associated birth defects. The lack of embryonic defects with Rasgrp3 loss of function under normal conditions (the present study and Roberts et al19) indicates that blockade of RasGRP3 is not detrimental to overall development. The focus of the present study was analysis of gain of function and loss of function for RasGRP3 in developing vessels and endothelial cells, and we have shown that RasGRP3-mediated signaling uses Ras/ERK as a target and mediates ET1 effects on angiogenesis. It will be interesting to determine whether RasGRP3-mediated signaling is important in adult vascular pathologies.

Acknowledgments
We acknowledge Dr Channing Der and Dr Natalia Mitin for productive discussions and the CAAX construct.

Sources of Funding
This work was supported by grants from the National Institutes of Health, National Heart, Lung, and Blood Institute (HL83262 and HL43174; V.L.B.), an American Heart Association predoctoral Fellowship (P.K.R.), a University of North Carolina Undergraduate Summer Fellowship (J.Y.H.), predoctoral fellowships from the National Science Foundation and Department of Defense (D.M.R.), and a National Institutes of Health–National Institute of Child Health & Human Development training grant (J.H.F.; T32 HD46369).

Disclosures
None.

References
4. Randhawa et al RasGRP3 Mediates Diabetic Embryonic Vasculopathy 1207


The Ras activator RasGRP3 mediates birth defects associated with diabetes. Maternal diabetes leads to elevation of diacylglycerol and endothelin and blood vessel dysfunction. RasGRPP3 regulates cytoskeletal organization and morphology by modulating endothelial cell division. RasGRP3 protects mouse embryos from diabetes-induced birth defects in blood vessels and other tissues. Endothelial cells that expressed activated RasGRP3 had elevated levels of active Ras and ERK. Endothelial cells that lacked RasGRP3 did not activate Ras or ERK in response to a surrogate of diacylglycerol, PMA (phorbol 12-myristate 13-acetate) or endothelin-1. Endothelin-mediated endothelial cell proliferation, migration, and blood vessel formation were attenuated in vessels that lacked RasGRP3. Thus, RasGRP3 is necessary and sufficient for the activation of Ras signaling in endothelial cells and in developing blood vessels in response to signals that are elevated in diabetes. Moreover, RasGRP3 is required for the full spectrum of vascular and nonvascular birth defects induced by diabetes. Our work for the first time demonstrates a critical role for RasGRP3 in the complications of diabetes and reveals a link between RasGRP3 and endothelin-mediated signaling. It also defines Ras as a RasGRP3 target in endothelial cells. This work advances our understanding of pathways that mediate diabetic complications and is significant in the suggestion that RasGRP3 may be a novel therapeutic target for fetal complications induced by diabetes.
The Ras Activator RasGRP3 Mediates Diabetes-Induced Embryonic Defects and Affects Endothelial Cell Migration

Circ Res. 2011;108:1199-1208; originally published online April 7, 2011; doi: 10.1161/CIRCRESAHA.110.230888
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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1. DETAILED METHODS

Diabetic mice

Female mice (C57Bl6/J purchased from Jackson Laboratories or Rasgrp3gt/gt mice backcrossed to N8 on the C57Bl6/J background) at 6-8 weeks of age were made diabetic following the protocol in “Animal Models of Diabetic Complications Consortium”. Mice were fasted and injected IP with streptozotocin (STZ) (stored at -20°C and dissolved to a final concentration of 7.5 mg/mL in 0.1M Na-Citrate buffer (pH 4.5) immediately prior to injection), at a dose of 50 mg STZ/kg of body weight, daily for 5 days. Controls were injected with citrate buffer alone. Alternatively, Ins2Akita/+ mice on the C57Bl6/J background (Jackson Laboratories) were bred to obtain Ins2Akita/+; Rasgrp3gt/gt mice. Blood glucose was monitored weekly using a OneTouch Ultra device and a drop of blood from a tail nick. Mice were considered diabetic with blood glucose levels over 250mg/dL. Most mice were diabetic 2-3 weeks after the last injection and remained diabetic long-term. Mice were mated to genotype-matched males.

Embryo and yolk sac analysis

Embryos were harvested at E9.5, fixed in fresh 4% PFA (paraformaldehyde) overnight at 4°C, and whole mount stained for PECAM as described \(^1\). Briefly, embryos were washed 15 min in PBS, dehydrated through a methanol:PBS series, blocked in 5% H\(_2\)O\(_2\)/methanol 4 hr at RT, then stored overnight in 100% methanol at -20°C. They were rehydrated through a methanol:PBS series, blocked 2 x 1 hr in PBT (0.2%BSA, 0.1% Triton X-100 in PBS), incubated overnight at 4°C in 1:200 rat anti-mouse CD-31 (PECAM) primary antibody (BD Biosciences) in PBT, rinsed 5 x 1 hr in PBT and incubated overnight at 4°C in 1:200 goat anti-rat HRP-conjugated secondary antibody (Kirkegaard & Perry, cat. #474-1612) in PBT. Embryos were rinsed 5 x 1 hr in PBT, then developed for 20 min at RT in DAB solution (Vector Laboratories, cat. #SK-4100). After developing, embryos were rinsed 2 x 5 min in PBT, 2 x 5 min in PBS, and fixed overnight at 4°C in 2% PFA/0.1% glutaraldehyde in PBS. Embryos were rinsed 3 x 5 min in PBS, then stored in PBS at 4°C. Stained embryos were imaged with an Olympus SZH10 stereo microscope outfitted with a with a Olympus DP71 camera. Embryos were scored for developmental defects on a scale of 0-3, and defects included aberrant intersomitic vessels, lack of neural tube closure, perturbed somite patterning, lack of remodeling and excessive vessel coverage in the head plexus, gaps or excessive vessels dorsal to the somites, and overall body-axis deformities. Embryos with no defects were scored 0; embryos with a single minor defect were scored 1; embryos with several defects were scored 2; and embryos with the most comprehensive and severe defects were scored 3.

Yolk sacs were removed from the embryos after the rehydration step, and stained separately for PECAM as described \(^2\) with minor modifications. Briefly, yolk sacs were blocked in 5% goat serum, 1% BSA in PBST (PBS with 0.1% Triton 100X) overnight at 4°C, and then incubated in primary antibody (PECAM CD31, BD Pharmingen #557355, rat anti-mouse) at 1:200 in PBST overnight at 4°C. Yolk sacs were washed in SBT (PBS with 0.1% Triton 100X and 2.5% goat serum), 4 times over at least 4 hr. Yolk sacs were then incubated in secondary antibody (Alexa Fluor 488, Molecular Probes #A-11006, goat
anti-rat) overnight at 4°C and washed in SBT 4 times over at least 5 hr, and finally washed in PBS. Yolk sacs were then mounted flat on glass slides with Vectashield Hardest and allowed to harden, then imaged using an Olympus IX 50 Epifluorescence microscope.

Whole embryo culture
Whole embryo culture was performed as described, with minor modifications. Embryos were dissected into M2 media from maternal decidua with their yolk sacs intact. Dissections were performed at E7.5 (for PMA experiments) or E8.5 (for glucose experiments), and embryos were cultured for 24 hr in 25 mL Nunc screwcap tubes on rollers at 37°C. Culture media contained 50% rat serum, 50% Tyrode’s salt solution, and 0.1X penicillin/streptomycin and was sterile filtered using a 0.2 µM filter. A minimum of 0.75 mL of media was used per embryo. During culture, the media was treated at 12-hr intervals with a gas mixture containing 20% O₂ and 5% CO₂ and the tubes were sealed with vacuum grease. Some embryos were treated with 50nM PMA (phorbol 12-myristate 13-acetate) and some had 20 mM D-glucose added to the media during the culture period. After 24 hr, embryos were removed from the media and photographed.

Endothelial cells
HUVEC
RasGRP3 was amplified by PCR cloning from with appropriate restriction sites at the ends, cloned into pBSIISK+/CAAX vector (kindly provided by Dr. Channing Der), and verified by sequence analysis. This version was shuttled into pEGFP-C1 (Clonetech, Genbank #U55763) so that eGFP was linked to the N-terminus of RasGRP3-CAAX, and expression was from the CMV promoter. GFP-RasGRP3 was generated by excision of the CAAX domain using Hind III sites.

HUVEC (purchased from Clonetics) were cultured in EGM-2 medium (Lonza) on gelatin-coated dishes and used between passages 2-8. 1 x 10⁶ cells in HUVEC optimized buffer (Amaxa) were transfected with 5 µg DNA using a nucleofector (Amaxa, #VPB-1492) and HUVEC optimized kit, according to manufacturer’s directions. Fresh medium was added 2 hr post-transfection.

For staining, transfected HUVEC were plated in 4-chamber slides, and 48 hr post-transfection starved for 6 hr in HUVEC medium with 0.1% FBS and no growth factors. Cells were fixed in 3.7% formaldehyde for 12 min, permeabilized in PBS/0.1% Triton for 5 min, blocked in PBS/1% BSA for 20 min, and incubated with phalloidin-Alexa 555 (Molecular Probes) diluted 1:50 in blocking buffer for 20 min. Fixation and staining was at RT.

Migration assays were done with HUVEC at 48 hr post-transfection. Cells in slide flasks were labeled with Cell tracker Red (Molecular Probes), and imaged in green and red channels over a 5 hr period with a Nikon TE300 Inverted Microscope (Melville NY) with a Perkin Elmer spinning disc confocal head (Shelton CT) and outfitted with a heated stage. Images were collected at 1 min intervals using Metamorph software (version 6.3r5; Universal Imaging Corp, Downingtown PA) and a Hamamatsu Orca CCD camera.
Mouse endothelial cells

Mouse endothelial cells (wildtype and Rasgrp3^gt/gt^) were generated and expanded as described \(^4, 5\), with minor modifications. Lungs of P6 wildtype (WT) and Rasgrp3^gt/gt^ mice were minced and digested with collagenase (Type 1, #CLS-1, Worthington, Lakewood NJ), diluted to 2 mg/ml in PBS containing Ca\(^{++}\) and Mg\(^{++}\), for 1 hr at 37\(^{\circ}\)C, and then incubated with magnetic beads conjugated to anti-mouse PECAM antibody for 20 min at RT. Bead-selected cells were incubated with PyMT virus (2.5 x 10^7 cfu/ml, cells a gift of Dr. William Sessa) and 8 mg/ml polybrene diluted in complete EBM-2 medium (Bullet kit, Lonza CA) for 8 hr/day for 3 days; cells were allowed to recover in complete EBM-2 medium overnight between virus incubations. Following incubation, cells were treated with 500 mg/ml Geneticin (G418, CalBiochem) for 14 days. Drug-resistant clones were isolated using cloning rings and expanded. Cells were maintained in complete EBM-2 media and incubated at 37\(^{\circ}\)C in 5% CO\(_2\). Growth curves were generated by plating 5 x 10^3 cells/well, and trypsinized cells were counted daily using a hemocytometer.

Cells were washed in 1X PBS, fixed with 1:1 cold methanol-acetone or 4% paraformaldehyde (PFA) for 10 min, washed twice in 1X PBS, then blocked with staining media (3% FBS, 0.1% sodium azide in 1X PBS) for 1 hr at 37\(^{\circ}\)C. Cells were incubated in appropriate primary antibodies at 1:1000 in staining media overnight at 4\(^{\circ}\)C. Primary antibodies used were rat anti-mouse PECAM (MEC13.3, #553370, BD Biosciences), rat anti-mouse ICAM-2 (CD102, #553326, BD Biosciences), rat anti-mouse VE-cadherin (CD144, #550548, BD Biosciences), and rabbit anti-phospho-histone H3 (Ser10, #06-570, Millipore). Cultures were washed 2 X in 1X PBS, incubated in AlexaFluor488 goat anti-rat IgG (1:1000) or Alexa Fluor 594 donkey anti-rabbit IgG (#A21206, Invitrogen) diluted in staining media for 2 hr at RT, washed 2 X in 1X PBS and imaged using an inverted epifluorescence microscope (IX-50; Olympus) outfitted with a camera (DP71; Olympus), or a confocal microscope (LSM 5 Pascal, Carl Zeiss, Inc.) using PASCAL Release version 4.2 SP1 acquisition software (Carl Zeiss, Inc.).

Endothelial cells were harvested and RNA isolated using Trizol (Invitrogen). RNA was purified using the RNAeasy kit (Qiagen), and cDNA was generated as described \(^6\). Equivalent amounts of cDNA were amplified by RT-PCR using RasGRP3 primers (forward 5'-AGAGAACCTGCCTCGTAC-3'; reverse 5'-GTGTTGCCGCTTCGCGCT-3') and lacZ primers (forward 5'-TTGAAAATGGTCTGCTGCTG-3'; reverse 5 '-TTGGCTTCATCCACCACATA-3') as described \(^7\). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward 5'-AGCCCCATCACCATCTTTCC-3'; reverse 5'-GCCATCCAGTCTTCTGG-3') were used as a control.

For mitotic index determination, cells were plated at 2.5 x 10^4 cells in chamber slides, serum-starved in 1% FBS overnight, then stimulated with 100nM ET1 in 1% FBS for 24 hr. Cells were fixed with 1:1 methanol:acetone and stained for PECAM and PH3 as described. Ten images from each sample were taken at 10X magnification, and the mitotic index was calculated by counting the number of PH3-positive cells divided by the PECAM-positive cells. For migration assays, Boyden chambers were used as described.
Transwell chambers fitted with 8-µm pore size filter membranes (Corning Incorporated, Corning NY) were immersed in medium. Cell suspensions were prepared in 1% FBS, and 2 x 10^4 cells in 100 µl were plated in the upper chamber. Cells were incubated overnight at 37°C, then incubated with 600 µl of 1% FBS containing 2.5 nM VEGF-A, or 100 nM ET1, placed in the bottom of each transwell chamber for 6 hr at 37°C. Cells on the upper surface of the filter were removed, and cells on the bottom surface of the filter were fixed and stained with Hema 3 stain (#122-911, Fisher, Pittsburgh PA). Migration was quantified by counting the total number of cells in 10 fields per chamber at 20X magnification using an Olympus IX-50 epifluorescence microscope attached to a camera.

**ES cell differentiation**

Wild type (WT, +/+ ) and RasGRP3 deficient (Rasgrp3<sup>gt/gt</sup>, where <i>gt</i> represents a gene trap insertion in the RasGRP3 locus that results in a loss-of-function mutation)<sup>7</sup>) ES cells were maintained and differentiated in vitro as previously described<sup>9</sup>. For inhibitor studies, wild type ES cell cultures were differentiated to day 7, and then pre-treated with the appropriate inhibitor for 2 hr prior to PMA (phorbol 12-myristate 13-acetate) or ET1 addition. Cultures were stimulated with 100 nM PMA for 24 hr, and then fixed with 1:1 methanol/acetone and stored in PBS at 4°C. Unless otherwise stated, inhibitors remained on the ES cell cultures for the duration of PMA treatment. Dose curves ranging from 1 µM to 20 µM were performed, and the most effective concentration that maintained wild-type vessels was used. Inhibitor concentrations used were: FTI-2153 (farnesyl transferase inhibitor) at 10 µM, U0126 (MEK inhibitor) at 10 µM, SB203580 (p38 MAPK inhibitor) at 5 µM, SP600125 (JNK inhibitor) at 10 µM, Bisindolylmaleimide I (BIM; general PKC inhibitor) at 10 µM, G66976 (PKC<sub>α</sub> and PKC<sub>β</sub> inhibitor) at 6 µM, and rottlerin (PKC<sub>δ</sub> inhibitor) at 10 µM. Inhibitors were purchased from Calbiochem (San Diego, CA). For ET1 studies, ES cultures were treated with fresh medium containing 100 nM ET1 daily on days 5-8 or fresh medium containing 10 µM ET1 on day 7-8, then fixed with 1:1 methanol:acetone and stored in PBS at 4°C.

**Antibody staining**

Antibody staining of ES cell cultures was as previously described<sup>1, 9</sup>. Briefly, fixed ES cell cultures were blocked in staining media (3% fetal bovine serum and 1% sodium azide in 1X PBS) for 1 hr at 37°C. Cultures were then incubated with rat anti-mouse PECAM (Mec 13.3; BD Pharmingen, San Diego, CA) at 1:1000 in staining media for 1 hr at 37°C and subsequently washed 3 times with staining media. The cultures were incubated with goat anti-rat Alexa 488 (Molecular Probes, Eugene, OR) at 1:200 for 1 hr at 37°C, rinsed in staining medium 3 times, then stored in 1X PBS at 4°C. Staining was visualized using either an Olympus IX-50 inverted microscope and epifluorescence, or a Zeiss 510 confocal microscope.

**Quantitative image analysis**

Quantitative image analysis of PECAM-stained day 8 ES cell cultures was performed as previously described<sup>1</sup>. Briefly, 10X images of non-overlapping areas with complete cell coverage were acquired. In general, the imaged area of each well was more than 60% of the total area. Images were analyzed using Metamorph software to determine the percent area of PECAM staining normalized to controls. On average, three
to four wells of each condition were used for analysis. All values were compared by the two-tailed Student’s T test, and p values <0.05 were considered significant.

**Ras and ERK activation assays**

Ras and ERK activation assays were performed as described, with minor modifications\(^{10,11}\). HUVEC were grown to near confluency for 48 hr after transfection (50-80% transfected by GFP labeling), serum starved as described overnight, then put in lysis buffer (50 mM Tris (pH 7.4), 0.15 M NaCl, 1% Triton, 10% glycerol, 1 mM EDTA, 10mM MgCl\(_2\)) with protease inhibitors. Mouse endothelial cells were grown to 60% confluence, serum-starved in 0.1% FBS/EBM-2 media overnight, then left untreated or treated with 100 nM PMA, 5 nM VEGF-A, or 100 nM ET1 diluted in 0.1% FBS/EBM-2 medium for 30 min (PMA) or 5 min (VEGF-A, ET1) prior to lysis. Ras activation assays were performed using the Ras Activation Assay Kit (Upstate, Lake Placid, NY) per manufacturer’s protocol, and 10% of the lysate was used for total Ras determination. The remaining lysate was incubated with 10 \(\mu\)l of agarose beads conjugated to the Ras binding domain of Raf (RBD) for 1 hr at 4ºC, briefly centrifuged to pellet the beads, then washed with lysis buffer. Samples were suspended in 50 \(\mu\)l of 2X sample buffer and boiled for 5 min before loading on a 15% acrylamide gel for electrophoresis. Proteins were transferred to a PVDF membrane, blocked in 5% milk in 1X TBST (Tris-Buffered Saline with 0.1% Tween), and incubated with mouse monoclonal anti-Ras antibody (Clone Ras10, Upstate) at 1:1000 in blocking solution overnight at 4ºC. After washing 3 times for 15 min, the membrane was incubated for 2 hr at RT with a goat anti-mouse secondary antibody conjugated to HRP (Upstate) at 1:5000 in blocking solution. After washing the membrane was developed using ECL Plus according to the manufacturer’s instructions.

For phospho-ERK blots, most conditions were the same as for Ras assays, except cells were lysed in RIPA buffer and loaded on a 10% acrylamide gel. Primary antibody incubation was with rabbit anti-mouse pERK (Cell Signaling) at 1:1000 overnight at 4ºC, and total ERK was determined by incubation with mouse anti-mouse ERK1/2 (Abcam) at 1:1000 overnight at 4ºC. Secondary antibody incubation was with goat anti-rabbit HRP or goat anti-mouse HRP (Promega) at 1:5000 for 2 hr at RT. Blots were quantified using the integrated density function of ImageJ (Version 1.44a, NIH, USA) or a phosphoimager (Storm 840, Molecular Dynamics, Sunnyvale CA). Ras-GTP was first normalized to total Ras, then normalized to untransfected WT HUVEC. pERK intensities were divided by total ERK, then normalized to untransfected WT HUVEC.
Supplemental Figure I. Loss of Rasgrp3 protects from PMA-induced developmental perturbations. Mouse embryos that were WT (A-D) or mutant for Rasgrp3 (E-H) were dissected at E7.5 with yolk sac intact, and incubated for 24 hr in the presence of 50 nM PMA. WT embryos uniformly exhibited severe defects of the yolk sac and embryo proper, while Rasgrp3<sup>gt/gt</sup> embryos were relatively protected from PMA effects, with discernable anterior (a) and posterior (p) and allantois (arrows).

Supplemental Figure II. Loss of Rasgrp3 protects from glucose-induced defects. WT (A,B) or Rasgrp3<sup>gt/gt</sup> (C, D) embryos were dissected from diabetic mothers at E8.5 and cultured for 24 hr in control media (A, C) or media with 20 mM glucose (B, D), then processed for β-galactosidase activity. White arrows, normal blood vessels in head or intersomitic areas; black arrows, abnormal head or intersomitic vessels; black arrowheads, abnormal somitogenesis or axis formation.
Supplemental Figure III. Loss of Rasgrp3 protects from diabetes-induced defects.

WT (A,B) or Rasgrp3<sup>gt/gt</sup> (C, D) embryos were dissected from diabetic mothers at E9.5 and stained in whole mount for PECAM. (A, B) arrows point to severe defects seen in two different WT embryos; (C, D) two different Rasgrp3<sup>gt/gt</sup> embryos have normal development.

Supplemental Figure IV. Distribution of Embryo Severity Index values relative to genotype/condition. For each group, embryos were scored on a scale from 0 (no defects) to 3 (severe defects) as described. The proportion in each category is indicated. WT, mothers were normo-glycemic; STZ, mothers were treated with streptozotocin and were diabetic.
Supplemental Figure V. Lack of yolk sac vascular defects associated with genotype/condition. (A-F) Yolk sacs of E9.5 embryos were processed for PECAM (green) immunofluorescence. No significant trends were found that correlated with genotype, condition, or severity of embryo defects.
Supplemental Figure VI. Characterization of Rasgrp3^gt/gt endothelial cells. WT (A, C) and Rasgrp3^gt/gt (B, D) endothelial cells were isolated as described, and stained for PECAM (A, B) or VE-cadherin (C, D). (E) RT-PCR from RNA isolated from: lane 1, WT dy 8 ES cell cultures; lane 2, Rasgrp3^gt/gt dy 8 ES cell cultures; lane 3, WT endothelial cells; and lane 4, Rasgrp3^gt/gt endothelial cells. Appropriate primers amplified a RasGRP3 band in WT embryos and endothelial cells, while lacZ primers amplified the reporter inserted in the gene trap (gt) allele in Rasgrp3^gt/gt embryos and endothelial cells. (F) A growth curve showed similar kinetics for both WT and Rasgrp3^gt/gt endothelial cells.
Supplemental Figure VII. RasGRP3-dependent PMA-induced vessel dysmorphogenesis is blocked by MEK inhibition. Day 8 ES cell cultures stained for PECAM. (A, C, E, G) control cultures; (B, D, F, H) cultures incubated with PMA (100 nM) for 24 hr. (A,B) WT vessels with no inhibitor; (C,D) Rasgrp3<sup>gt/gt</sup> vessels with no inhibitor; (E,F) Vessels co-incubated with the MEK inhibitor U0126; (G,H) Vessels co-incubated with the p38 MAPK inhibitor SB203580; (I), Vessel area was quantified. ***, p ≤ 0.00001 for -PMA (lane 1) vs. +PMA (lane 2); ##, p ≤ 0.0001 for +PMA (lane 2) vs. +PMA,+MEK inhibitor (lane 4); N.S., p = 0.24 for +PMA (lane 2) vs. +PMA,+p38 MAPK inhibitor (lane 6).
Supplemental Figure VIII. RasGRP3-dependent PMA-induced vessel dysmorphogenesis is blocked by PKC inhibition. Day 8 ES cell cultures stained for PECAM. (A, C, E, G) control cultures; (B, D, F, H) cultures incubated with PMA (100 nM) for 24 hr. (A,B) Vessels with no inhibitor; (C,D) Vessels co-incubated with the general PKC inhibitor BIM; (E,F) Vessels co-incubated with the conventional PKC inhibitor Gö6976; (G, H) Vessels co-incubated with the PKC inhibitor rottlerin; (I), Vessel area was quantified. ***, p ≤ 0.00001 for -PMA (lane 1) vs. +PMA (lane 2); ##, p ≤ 0.001 for +PMA (lane 2) vs. +PMA, +BIM inhibitor (lane 4); ###, p ≤ 0.0001 for +PMA (lane 2) vs. +PMA, +Gö6976 inhibitor (lane 6); #, p ≤ 0.01 for +PMA (lane 2) vs. +PMA, +rottlerin (lane 8).
Supplemental Figure IX. RasGRP3-dependent Endothelin-1 mediated vessel dysmorphogenesis is blocked by PKC inhibition. Day 8 ES cell cultures stained for PECAM. (A-C) control cultures and (D-F) cultures incubated with the general PKC inhibitor BIM and co-incubated with (B, E) PMA, (C, F) ET1. Note the vessel dysmorphogenesis seen in panels B and C is abrogated in panels E and F, with PKC inhibition.
Supplemental Figure X. Original blots for Figure A-B. (A) The original blots used to set up Figure 4A. (B) Original blots used to set up Figure 4B.


