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The Ras activator RasGRP3 mediates diabetes-induced embryonic defects and affects endothelial cell migration

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Abstract

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 one or more pathways activated downstream of aberrant glucose metabolism, since poorly controlled maternal glucose levels correlate with the frequency and severity of the defects.

Objective—We asked whether RasGRP3, a Ras activator expressed in developing blood vessels, mediates diabetes-induced vascular developmental defects.

RasGRP3 is activated by diacylglycerol (DAG), and DAG is over-produced by aberrant glucose metabolism in diabetic individuals. We also investigated the effects of over-activation 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 lacking *Rasgrp3* function. Endothelial cells that expressed activated RasGRP3 had elevated Ras-ERK signaling and perturbed migration, while endothelial cells lacking *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.

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None.

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Conclusions—These findings provide the first evidence that RasGRP3 contributes to developmental defects found in embryos developing 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.

Keywords

blood vessel disruption; diabetic embryopathy; Ras activator; endothelial migration; actin cytoskeleton; endothelin-1

INTRODUCTION

Receptor-mediated signaling is required for endothelial cell proliferation and migration, processes that are critical to blood vessel formation and function [1]. Signaling downstream of receptor engagement in endothelial cells leads to the activation of several pathways, including PLCγactivation to produce diacylglycerol (DAG). DAG in turn activates downstream targets to affect endothelial cell behaviors. Signals that are up-regulated in diabetes, such as 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 [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 [3, 4]. It is assumed that these vascular defects are mediated, at least in part, by elevated DAG levels, since mouse embryos recovered from diabetic mothers had elevated DAG levels and increased developmental defects [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 [5-9]. DAG and phorbol esters are potent activators of the PKC family of proteins. Numerous mammalian PKC isoforms fall into several sub-families, and DAG/phorbol esters activate PKC signaling by binding to C1 domains found in the classic (PKCα, β, γ) and novel (PKCδ, ε, η, θ) sub-groups of PKCs [10]. Recently several non-PKC protein families were identified that contain C1 domains and respond to DAG and phorbol esters, suggesting that some DAG/phorbol ester-mediated responses require these proteins [11].

RasGRPs are non-PKC DAG/phorbol ester receptors that function as guanine nucleotide exchange factors (GEFs) and activate the Ras family of GTPases [12, 13]. Ras proteins are localized to membranes, and their activation is controlled in part by proximity to GEFs, such that recruitment of GEFs 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 MAPK, and JNK. Ras proteins are activated by phorbol esters in cultured endothelial cells [14], 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 non-redundant 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 [15-17]. RasGRP2 was recently reported to have effects on vascular development in *Xenopus* [18]. We identified RasGRP3 in a murine-based gene-trap screen as a locus expressed in endothelial cells of

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developing vessels and required for mediating the endothelial cell effects of DAG/phorbol esters [19]. The expression profile and DAG/phorbol ester interactions suggested that RasGRP3 might mediate the effects of excess DAG on developing vessels in diabetes.

RasGRP3 activates Ras, Rap, and R-ras in vitro and in mouse embryo fibroblasts [20]. In addition to requiring DAG/phorbol ester activity for membrane localization [21], RasGRP3 is phosphorylated by PKC at Thr133, and this phosphorylation is required for RasGRP3 activity in B cells [22, 23]. RasGRP3 also binds dynein light chain, a component of a microtubule plus-end directed motor, but the significance of this binding in vivo is not clear [24]. We showed that RasGRP3 is expressed in angiogenic vessels. Although a genetic loss-of-function generated by the gene trap did not affect development, embryonic vessels exposed to phorbol ester exhibited dysmorphogenesis that required *Rasgrp3* function, identifying RasGRP3 as a novel endothelial phorbol ester receptor [19]. Here we further characterize the role of RasGRP3-mediated Ras signaling in embryos, endothelial cells, and developing vessels. We find that loss of *Rasgrp3* function significantly reduces diabetes-induced birth defects in vivo, including embryonic vascular defects. We show that Ras is a target of RasGRP3 in endothelial cells, and that RasGRP3 is required for ET1-mediated effects on vessel morphogenesis. Our data are consistent with a model in which RasGRP3 signaling in embryonic endothelial cells transduces signals downstream of DAG that affect endothelial cell behaviors and lead to vessel dysmorphogenesis. Thus excess DAG in diabetes likely over-activates RasGRP3, and this contributes to the perturbed development of fetuses in diabetic environments.

MATERIALS AND METHODS

Diabetic mice and embryo analysis

Female mice (C57Bl/6J purchased from Jackson Laboratories, or *Rasgrp3*$^{gt/gt}$ (a loss-of-function null mutation [19]) backcrossed to N8 on the C57Bl/6J background) at 6-8 weeks of age were made diabetic following the protocol in “Animal Models of Diabetic Complications Consortium”. Alternatively, *Ins2*^{Akita}/+ mice on the C57Bl6/J background (Jackson Laboratories, #003548) were bred to obtain *Ins2*^{Akita}/+; *Rasgrp3*$^{gt/gt}$ mice. Blood glucose was monitored weekly, and mice with blood glucose levels over 250 mg/dL were considered diabetic. Mice were mated to genotype-matched males, embryos were harvested at E9.5, fixed, and whole mount stained for PECAM as described [25]. Stained embryos were imaged and scored for defects as described (Supplemental Methods).

Whole embryo culture was done on embryos dissected at E7.5 or E8.5, in roller bottles as described [26]. Some embryos were treated with 50nM PMA (phorbol 12-myristate 13-acetate) and some with 20 mM glucose during the culture period. After 24 hr, embryos were removed and photographed.

Endothelial cells

HUVEC were purchased from Clonetics, cultured according to manufacturer's protocol, and used between passages 2-8. Transfections were done using an Amaxa nucleofector according to directions. For staining, cells were fixed, permeabilized, blocked, and incubated with phalloidin-Alexa 555 (Molecular Probes). Migration assays were carried out 48 hr post-transfection as described (Supplemental Methods). Average velocity and distance to origin of cells was calculated using trajectory measures as diagrammed in Fig. 3J.

Mouse endothelial cells (wildtype and *Rasgrp3*$^{gt/gt}$) were generated and expanded as described [27, 28], with minor modifications. Proliferation assays and migration assays were as described (Supplemental Methods).

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ES cell differentiation and analysis

Wild type (WT, $^{+/+}$) and RasGRP3 deficient (*Rasgrp3$^{gt/gt}$*) ES cells were maintained and differentiated for 8 days as previously described [29]. For inhibitor studies, day 7 ES cell cultures were pre-treated with the appropriate inhibitor for 2 hr prior to addition of 100 nM PMA for 24 hr. For ET1 stimulation, 100 nM ET1 in fresh medium was added daily between days 5-8. Antibody staining of ES cell cultures was as previously described [25, 29]. Quantitative image analysis of PECAM-stained ES cell cultures was performed as previously described [25].

Ras and ERK activation assays

HUVEC (50-80% transfected by GFP labeling after transfection) or mouse endothelial cells were grown to near confluency for 48 hr, serum starved overnight, then processed as described (Supplemental Methods) for Ras-GTP immunoprecipitation, or total Ras, pERK, 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 mid-gestation mouse embryos [19], we reasoned that embryos exposed to elevated DAG signaling would be susceptible to effects of RasGRP3-mediated signaling in vivo. Thus we examined the effects of elevated DAG/phorbol ester, glucose, and the diabetic environment on these stages of mouse development. We first mimicked elevated DAG signaling by incubating mouse embryos harvested at E7.5 for 24 hr. in phorbol ester under whole embryo culture conditions (Online Fig. I). WT embryos exposed to PMA were severely affected (Online Fig. I, A-D), with loss of anterior-posterior landmarks, and yolk sacs with large cavities. In contrast, *Rasgrp3$^{gt/gt}$* mutant embryos incubated under the same conditions were surprisingly intact, and anterior-posterior landmarks were evident, along with intact yolk sacs (Online Fig. I, E-H). We next incubated embryos in 20 mM glucose for 24 hr to mimic the diabetic environment (Online Fig II). Glucose exposure between E8.5 and E9.5 led to vascular defects in WT embryos, and likely non-vascular defects such as perturbed somitogenesis and axis defects as well. In contrast, *Rasgrp3$^{gt/gt}$* mutant embryos were relatively refractory to the teratogenic effects of elevated glucose.

To more precisely define the effects of a diabetic environment on developing embryos, we induced diabetes in female mice, then set up matings and examined embryos. On an outbred background, WT embryos often showed quite severe defects, including aberrant somite formation and vessel patterning, relative to *Rasgrp3$^{gt/gt}$* mutant embryos (Online Fig. III). To make more rigorous comparisons, we next examined embryos on the C57Bl6/J inbred background. We induced diabetes chemically via STZ, and we also utilized mice carrying the *Ins2^{Akita}* mutation that induces diabetes genetically. We monitored the average maternal blood glucose at sacrifice via tail bleed, and found the following values: WT STZ, 381 mg/dl; *Rasgrp3$^{gt/gt}$* STZ, 448 mg/dl; *Ins2^{Akita}; Rasgrp3$^{gt/gt}$* 387 mg/dl). Embryos were harvested from diabetic mothers at E9.5, whole-mount stained for PECAM to visualize vessel development and patterning, and the severity of defects in head plexus vessels, intersomitic vessels, and trunk/somite scored as described in the Methods (Fig. 1). WT embryos from diabetic mothers induced either chemically or genetically had a significantly elevated developmental severity index compared to controls (Fig. 1 A-B, E, O; Online Fig. IV). Closer examination of the somite region showed defects in somites and in the pattern of intersomitic vessels (Fig. 1 G-J), and similar defects were seen in the vascular plexus of the head (Fig. 1 K-N). In contrast, embryos similarly developing in diabetic mothers but genetically deficient for *Rasgrp3* appeared relatively unaffected, and their developmental

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severity index was significantly lower than WT counterparts, and close to control levels (Fig. 1, C-N, O; Online Fig. 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 Fig. V and data not shown). Thus loss of *Rasgrp3* function has a protective effect on diabetes-induced developmental defects, including vascular defects, suggesting that DAG-mediated activation of RasGRP3 mediates 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 HUVEC that up-regulated RasGRP3 activity, because the developmental defects are predicted to result from over-activation of RasGRP3 by DAG (Fig. 2). Wild-type RasGRP3 linked to a GFP reporter, and constructs in which a K-Ras CAAX or H-Ras CAAX sequence was linked to the protein, were transiently expressed in HUVEC (Fig. 2A). The CAAX sequences localize RasGRP3 to membranes [20], 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 over-expression effects. Over-expression of WT RasGRP3 did not significantly increase Ras activation over baseline, but both of the CAAX tagged RasGRP3 proteins stimulated significant Ras activation (Fig. 2B, C). ERK activation is downstream of Ras activation, and in HUVEC both CAAX-tagged RasGRP3 proteins also significantly induced ERK activation (Fig. 2B, C). 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 over-activation on the cytoskeleton, since in other cell types Ras activation affects the cytoskeleton [30, 31]. HUVEC expressing the constructs described above were stained for microtubules (α-tubulin, data not shown) and actin (phalloidin) (Fig. 3A-H). The microtubule staining pattern was not affected by over-expression of RasGRP3 (data not shown); however, the actin cytoskeleton was dramatically altered in endothelial cells over-expressing membrane-localized RasGRP3 (Fig. 3E-H). RasGRP3-CAAX expressing cells appeared larger and flatter, and they had very few actin stress fibers. Cortical actin was also diminished in endothelial cells over-expressing RasGRP3-CAAX. Since actin stress fibers are implicated in proper migration, we investigated the migratory behavior of endothelial cells over-expressing RasGRP3 (Fig. 3I-L). Endothelial cells over-expressing RasGRP3 were identified by GFP expression, and all cells were labeled with cell-tracker (Fig. 3I). Cells were imaged, and the average velocity and distance migrated from origin was calculated as described in methods (Fig. 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 (Fig. 3K-L). These results show that endothelial cells that over-express membrane-localized (and thus activated) RasGRP3 have a perturbed actin cytoskeleton, and an attenuated ability to migrate in a forward direction.

To investigate the effects of loss-of-function for *Rasgrp3* on signaling pathway(s), endothelial cells were isolated and immortalized from WT and *Rasgrp3*$^{gt/gt}$ mice. The cells were expanded clonally, and the endothelial identity of the cells verified by expression of endothelial markers. The cell lines were greater than 95% positive for PECAM, VE-cadherin, and ICAM-2 (Online Fig. VI, A-D and data not shown). RT-PCR analysis showed loss of RasGRP3 expression in *Rasgrp3*$^{gt/gt}$ endothelial cells, and growth curves showed that WT and *Rasgrp3*$^{gt/gt}$ endothelial cell grew at similar rates (Online Fig. VI, E-F).

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We asked whether RasGRP3 was necessary for Ras and ERK activation downstream of DAG/phorbol ester stimulation. WT and *Rasgrp3*$^{gt/gt}$ 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, *Rasgrp3*$^{gt/gt}$ endothelial cells stimulated with PMA showed no detectable Ras activation, and ERK activation was attenuated (Fig. 4A, Online Fig. 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 RasGRP3 activation in endothelial cells of developing vessels, utilizing a mouse embryonic stem (ES) cell differentiation model that supports the formation of primitive blood vessels in vitro via a programmed differentiation [29, 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* function [19] (Online Fig. VII, A-D). We therefore asked whether signaling downstream of activated RasGRP3 in developing vessels utilized the Ras effector pathways MEK/ERK, p38 MAPK or JNK. The vessel dysmorphogenesis seen upon PMA stimulation was significantly attenuated in the presence of the MEK inhibitor U0126 (Online Fig. VII, E-F, I). In contrast, the p38 MAP kinase inhibitor SB203580 had no effect on PMA-induced vessel dysmorphogenesis (Online Fig. VII, G-H, 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 GTPases.

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 Fig. VIII). A general inhibitor of most PKC isoforms, BIM (bisindolymaleimide), 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 Fig. VIII, C-D, I). Likewise, an inhibitor of the conventional PKC isoforms α and β, Gö6976, completely blocked PMA-induced vessel dysmporphogenesis (Online Fig. VIII, E-F, I). A third PKC inhibitor, rottlerin, predominantly affects PKCδ, and it partially blocked PMA-induced vessel dysmorphogenesis (Online Fig. VIII, G-H, 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.

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 signal(s) 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, two angiogenic factors that utilize DAG-Ras-ERK downstream signaling. WT and *Rasgrp3*$^{gt/gt}$ endothelial cells were stimulated with VEGF or ET1, and levels of active Ras and ERK analyzed (Fig. 4B-C, Online Fig. X, B). As predicted, WT endothelial cells had increased levels of active Ras and ERK upon treatment with either VEGF or ET1. In contrast, while *Rasgrp3*$^{gt/gt}$ 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.

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RasGRP3 is required for ET1-induced stimulation of endothelial cell proliferation and migration

Activation of Ras-ERK signaling by ET1 stimulates endothelial cell proliferation and migration [34]. To determine whether ET1-induced endothelial cell proliferation and migration were RasGRP3-dependent, WT and *Rasgrp3*$^{gt/gt}$ endothelial cells were treated with ET1 and evaluated. WT endothelial cells exhibited over a 2-fold increase in mitotic index over baseline with ET1 treatment, while *Rasgrp3*$^{gt/gt}$ endothelial cells did not exhibit a significant change in their mitotic index with ET1 treatment (Fig. 5A-E). In a Boyden chamber migration assay, VEGF induced migration of both WT and *Rasgrp3*$^{gt/gt}$ endothelial cells significantly and to a similar degree. However, ET1 only stimulated migration of WT endothelial cells, and did not significantly induce migration of *Rasgrp3*$^{gt/gt}$ endothelial cells (Fig. 5F). These results indicate that ET1-induced proliferation and migration require *Rasgrp3* function, whereas VEGF-induced migration occurs independent of RasGRP3.

RasGRP3 mediates vessel dysmorphogenesis induced by ET1

Since both ET1-induced endothelial cell proliferation and migration are RasGRP3-dependent, we asked whether ES cell-derived blood vessels had RasGRP3-dependent dysmorphogenesis induced by ET1 (Fig. 6). ET1 treatment of WT vessels led to loss of the fine vascular network and significantly increased vascular area (Fig. 6A-B, E). This response was *Rasgrp3*-dependent, as *Rasgrp3*$^{gt/gt}$ vessels retained the fine vascular network and did not exhibit increased vascular area under with ET1 treatment (Fig. 6C-D, E). ET1-mediated vessel dysmorphogenesis was also dependent on PKC activity, since PKC inhibition blocked ET1 effects on vessels (Online Fig. IX). Thus developing vessels exhibit vessel dysmorphogenesis in response to ET1, and that response requires RasGRP3 and PKC function. Taken together, these data suggest 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 required for the DAG/phorbol ester-mediated responses of these vessels. Here we posit that RasGRP3 is over-activated in embryos developing in a diabetic environment and susceptible to birth defects, and we show that loss of RasGRP3 significantly attenuates the detrimental effects of a diabetic environment on embryonic vascular development. We also define the molecular and cellular processes perturbed by DAG/phorbol ester-induced activation of RasGRP3 activation in endothelial cells of developing vessels. We define ET1 as an upstream input and Ras as a target of RasGRP3 in endothelial cells, and we show that manipulation of RasGRP3 perturbs endothelial migration. These data lead 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 (Fig. 7).

We activated RasGRP3 in developing vessels using phorbol ester as a DAG mimic, and RasGRP3-dependent vessel dysmorphogenesis required MEK/ERK signaling. Our data indicates that Ras is a primary target of RasGRP3 in endothelial cells, since over-expression of RasGRP3 linked to either K-Ras CAAX or H-Ras CAAX domains significantly activated both Ras and ERK, while loss-of-function analysis showed that RasGRP3 was required for ET1- or DAG/phorbol ester-mediated Ras and MEK/ERK activation. Our 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 by PKC phosphorylation [12]. Over-expression of RasGRP3 alone was not sufficient to activate endothelial Ras, but over-expression of membrane localized forms was sufficient

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for activation, suggesting that endogenous PKC levels are not rate-limiting for RasGRP3 activation in endothelial cells. Our studies also reveal a requirement for classical/novel PKC isoform activity in the response of developing vessels to DAG/phorbol esters and ET1. While 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 [22, 23].

Elevated RasGRP3 activity affects specific cellular responses of endothelial cells. Endothelial cells that over-express activated RasGRP3 are flattened relative to controls, a phenotype also observed in neural cells expressing activated RasGRP3 [20]. 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 over-express RasGRP3 is similar to actin perturbations seen in tumor cells that express activated Ras [35-37]. 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 [30, 31, 38]. Actin stress fibers link to focal adhesions on the ventral side of cells, and this linkage promotes acto-myosin contractility [39, 40]. In terms of migration, stress fibers are thought to act as rudders that keep cells migrating directionally [41, 42]. Consistent with this idea, we find that endothelial cells over-expressing 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 over-expression of activated RasGRP3 mimics Ras over-activation 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 milieus [43] and leads to DAG production, so perhaps two 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 up-regulated by VEGF-A [19]. Several studies reported that ET1 angiogenic effects required VEGF-A [44, 45]. 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 two 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 lacking *Rasgrp3* are less susceptible to diabetes-induced birth defects, and in fact embryos lacking *Rasgrp3* were significantly protected from developmental defects produced in a diabetic fetal environment. This was true whether the mothers were diabetic from STZ destruction of pancreatic β cells or via the *Ins2^{Akita}* mutation, which inactivates the insulin II gene and thus leads to diabetes [46]. The embryonic defects we documented were similar to the defects reported by others [3, 4]. Interestingly, embryos that lacked *Rasgp3* function and developed in diabetic mothers had significantly fewer defects in developing somites and developing vessels, two embryonic organs that express RasGRP3 [19]. The vascular defects seen in WT embryos from diabetic mothers were consistent with the endothelial migration defects observed in primary endothelial cells, since the intersomitic

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vessels were sometimes blunted, with expanded migratory fronts. Interestingly, while yolk sac vessels showed some pattern defects, these did not significantly correlate with embryo genotype, diabetic environment, or overall embryo defects, in contrast to another group who described significant diabetes-induced yolk sac vasculopathy [4, 47]. 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 [3], indicating that upstream requirements for RasGRP3 activation are in place in the embryo. In our work, mothers lacking RasGRP3 became diabetic, and their average blood glucose at sacrifice was higher than WT controls. Whole embryo culture using medium supplemented with PMA showed that embryos lacking *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, showing 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 (this study and [19]) indicates that blockade of RasGRP3 is not detrimental to overall development. The focus of this study was analysis of gain- and loss-of-function for RasGRP3 in developing vessels and endothelial cells, and we show 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NON-STANDARD ABBREVIATIONS

RasGRP3	Ras guanyl-releasing protein 3
DAG	diacylglycerol
PLCγ	phospholipase C gamma
VEGF-A	vascular endothelial growth factor-A

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ET1	endothelin-1
PKC	protein kinase C
GEF	guanine nucleotide exchange factor
p38 MAPK	p38 map kinase
JNK	C-jun N-terminal kinase
PECAM	platelet endothelial cell adhesion molecule
PMA	phorbol 12-myristate 13-acetate
WT	wild-type
HUVEC	human umbilical vein endothelial cells
GFP	green fluorescent protein
STZ	streptozotocin
VE-cadherin	vascular endothelial-cadherin
ICAM-2	intercellular adhesion molecule-2

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NOVELTY AND SIGNIFICANCE

What is known?

--Diabetes leads to elevation of diacylglycerol and endothelin and blood vessel dysfunction

--Maternal diabetes leads to birth defects

--Diacylglycerol activates RasGRP3 (Ras guanyl releasing protein 3) a mediator of cell signaling

What new information does this article contribute?

--The Ras activator RasGRP3 mediates birth defects associated with diabetes

--RasGRP3 affects the migration of endothelial cells that form blood vessels

--RasGRP3 is required for major vessel responses to diacylglycerol and endothelin

This study focused on the Ras activator, RasGRP3, in blood vessel disruptions induced by diabetes. We show that a lack of RasGRP3 protects mouse embryos from diabetes-induced birth defects in blood vessels and other tissues. Endothelial cells expressing activated RasGRP3 had elevated levels of active Ras and ERK. Endothelial cells lacking 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 lacking 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 non-vascular birth defects induced by diabetes. Our work for the first time demonstrates a critical role for RasGRP3 in the complications of diabetes, and 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 suggesting that RasGRP3 may be a novel therapeutic target for fetal complications induced by diabetes.

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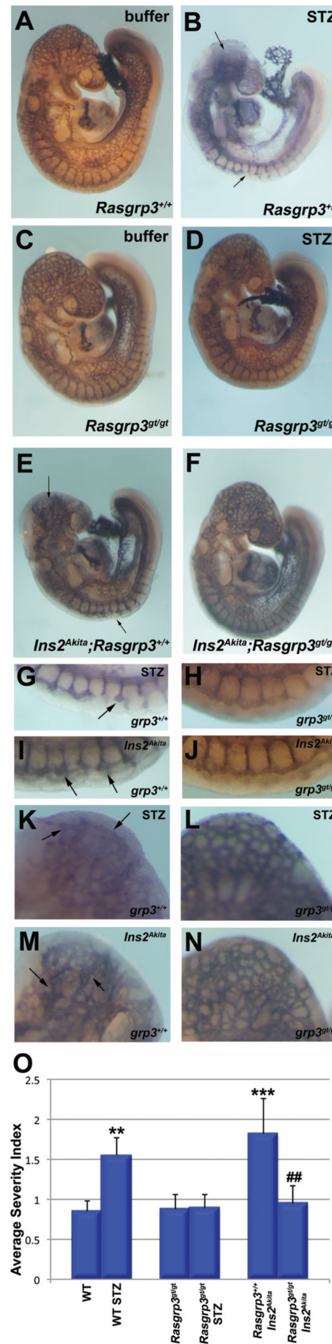

Figure 1. Loss of RasGRP3 attenuates diabetes-induced developmental defects

WT (A, B, G, K) and *Rasgrp3$^{gt/gt}$* (C, D, H, L) E9.5 embryos were isolated from mothers made diabetic with STZ (B, D, G, H, K, L), or isolated from *Ins2^{Akita}* (E, I, M) or *Ins2^{Akita}; Rasgrp3$^{gt/gt}$* (F,J, N) diabetic mothers and stained for PECAM. (A) WT embryos from non-diabetic WT mothers have few defects, while (B) WT embryos from diabetic mothers have defects in the somitic vasculature (arrow), head plexus (arrow), and somites; (C) *Rasgrp3$^{gt/gt}$* embryos from *Rasgrp3$^{gt/gt}$* non-diabetic mothers have few defects, and (D) *Rasgrp3$^{gt/gt}$* embryos from *Rasgrp3$^{gt/gt}$* diabetic mothers are also relatively normal. (E) Embryos from *Ins2^{Akita}; Rasgrp3$^{+/+}$* mothers have somitic vessel and head plexus defects (arrows), whereas (F) embryos from *Ins2^{Akita}; Rasgrp3$^{gt/gt}$* mothers have few defects. (G-J)

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Close-up of somitic regions of embryos of indicated genotypes; (K-N) close-up of head plexus regions of embryos of indicated genotypes. Arrows point to defects in vascular pattern. (O) The developmental severity index was calculated on groups of embryos as described in Methods. Mother's genotype and diabetic status: Lane 1, WT non-diabetic (n = 46); Lane 2, WT STZ diabetic (n = 51); Lane 3, *Rasgrp3*$^{gt/gt}$ non-diabetic (n = 25); Lane 4, *Rasgrp3*$^{gt/gt}$ STZ diabetic (n = 27); Lane 5, *Ins2*^{Akita}*; Rasgrp3*$^{+/+}$ diabetic (n = 17); Lane 6, *Ins2*^{Akita}*; Rasgrp3*$^{gt/gt}$ diabetic (n = 19). **, p 0.001 for WT vs. WT-STZ diabetic; ***, p 0.00001 for WT vs. *Ins2*^{Akita}; ##, p 0.0001 for *Ins2*^{Akita}*; Rasgrp3*$^{+/+}$ vs. *Ins2*^{Akita}*; Rasgrp3*$^{gt/gt}$.

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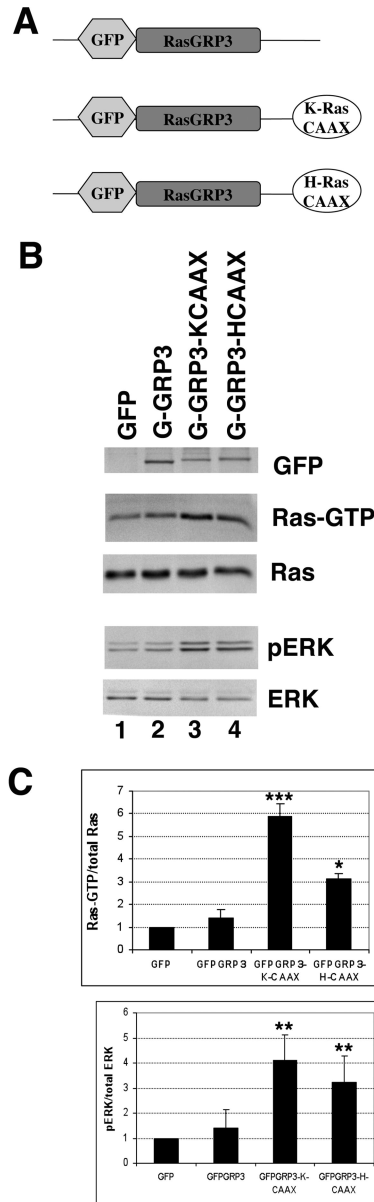

Figure 2. Over-expression of activated RasGRP3 activates Ras and ERK in endothelial cells

(A) HUVEC were transiently transfected with the constructs shown. (B) IB for GFP linked to RasGRP3 constructs (top panel) shows relative expression of the introduced genes; Ras-GTP panel is a Ras blot of Ras-RBD (Raf Binding Domain) immunoprecipitation to show activated Ras; Ras panel is 10% loading of input to show total Ras; pERK panel is blot hybridized with p-ERK (activated); ERK is total ERK blot. Lane 1, HUVEC transfected with GFP control; Lane 2, HUVEC transfected with GFP-RasGRP3; Lane 3; HUVEC transfected with GFP-RasGRP3-KCAAX; Lane 4; HUVEC transfected with GFP-RasGRP3-HCAAX. (C) Relative increase in reactivity for active Ras/total Ras (top panel) or active ERK/total ERK (bottom panel) relative to GFP control. *, p 0.01; **, p 0.002; ***, p 0.0001, relative to the relevant control.

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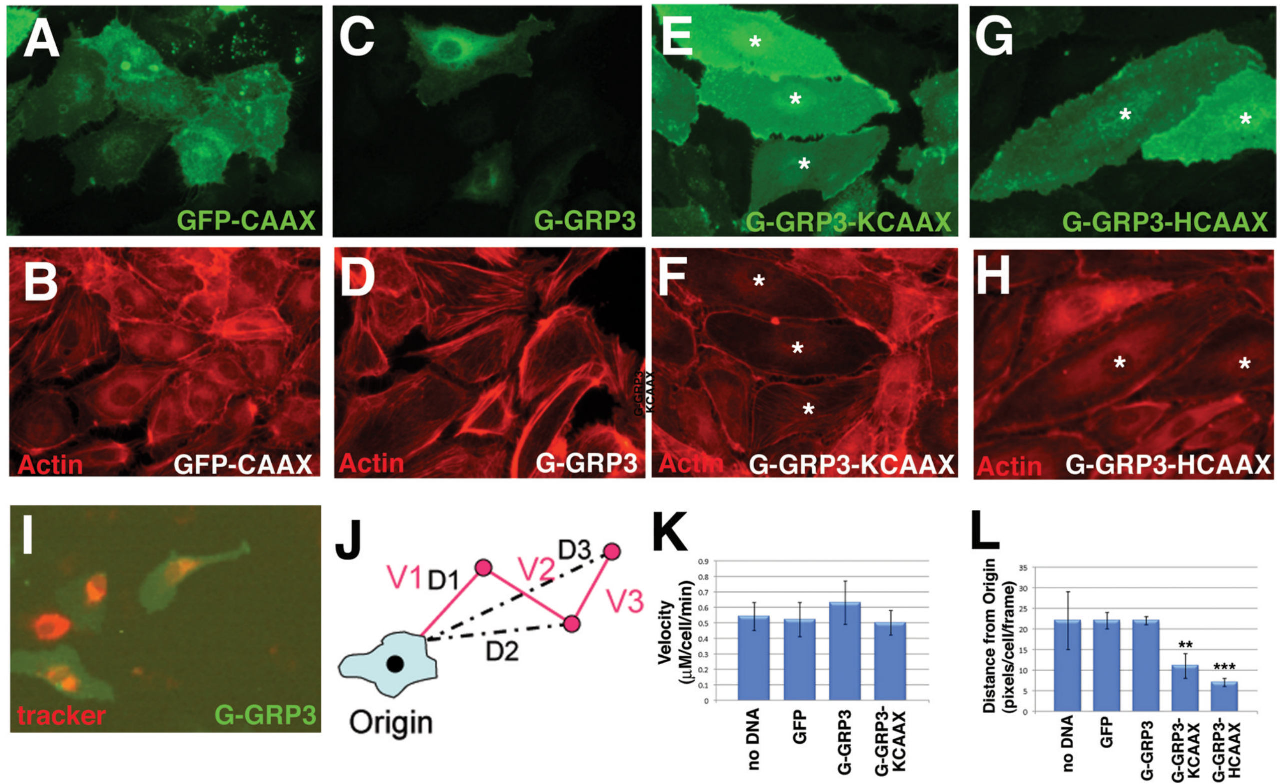

Figure 3. Over-expression of active RasGRP3 perturbs the actin cytoskeleton and migration of endothelial cells

(A-H), HUVEC transfected with the indicated constructs were incubated for 48 hr, then fixed and imaged for GFP (green) (A-D) or stained with phalloidin (red) (B-H). (A-B) control GFP-CAAX; (C-D) GFP-RasGRP3; (E-F) GFP-RasGRP3-KCAAX; (G-H) GFP-RasGRP3-HCAAX. Astericks in panels E-H indicate transfected cells with perturbed phalloidin staining. (I-J), HUVEC were labeled with cell-tracker and live imaged. (I) HUVEC expressing GFP-RasGRP3 (green) and labeled with celltracker (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 0.005; ***, p 0.0001, relative to control.

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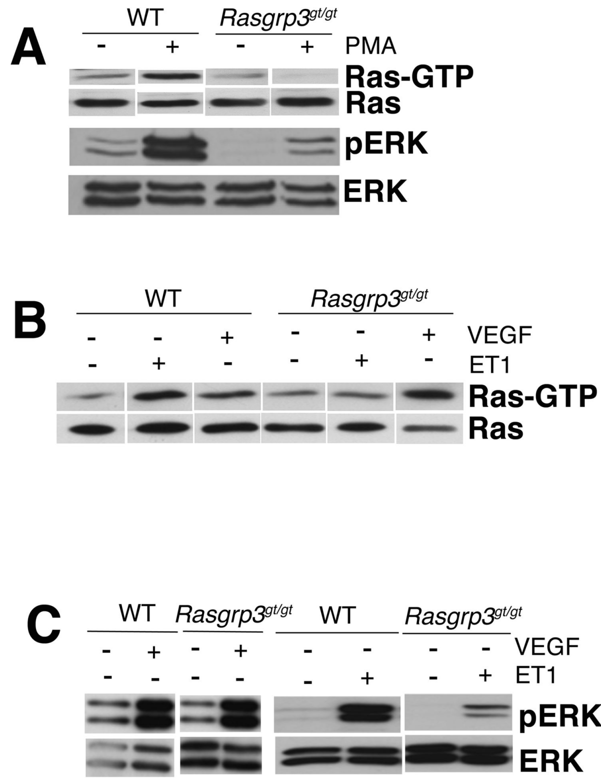

Figure 4. RasGRP3 is required for Ras-ERK activation downstream of phorbol ester and ET1 but not VEGF-A

(**A**) WT and *Rasgrp3$^{gt/gt}$* endothelial cells were treated with PMA and processed for Ras and ERK activation. Compared to WT endothelial cells, *Rasgrp3$^{gt/gt}$* endothelial cells did not show increased activated Ras (Ras-GTP) and had an attenuated activation of pERK. (**B**) WT and *Rasgrp3$^{gt/gt}$* 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 *Rasgrp3$^{gt/gt}$* endothelial cells had increased activated Ras (Ras-GTP) in response to VEGF-A but an attenuated response to ET1. (**C**) WT and *Rasgrp3$^{gt/gt}$* 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 *Rasgrp3$^{gt/gt}$* endothelial cells had increased pERK in response to VEGF-A but not in response to ET1. Experiments are representative of at least 3 replicates.

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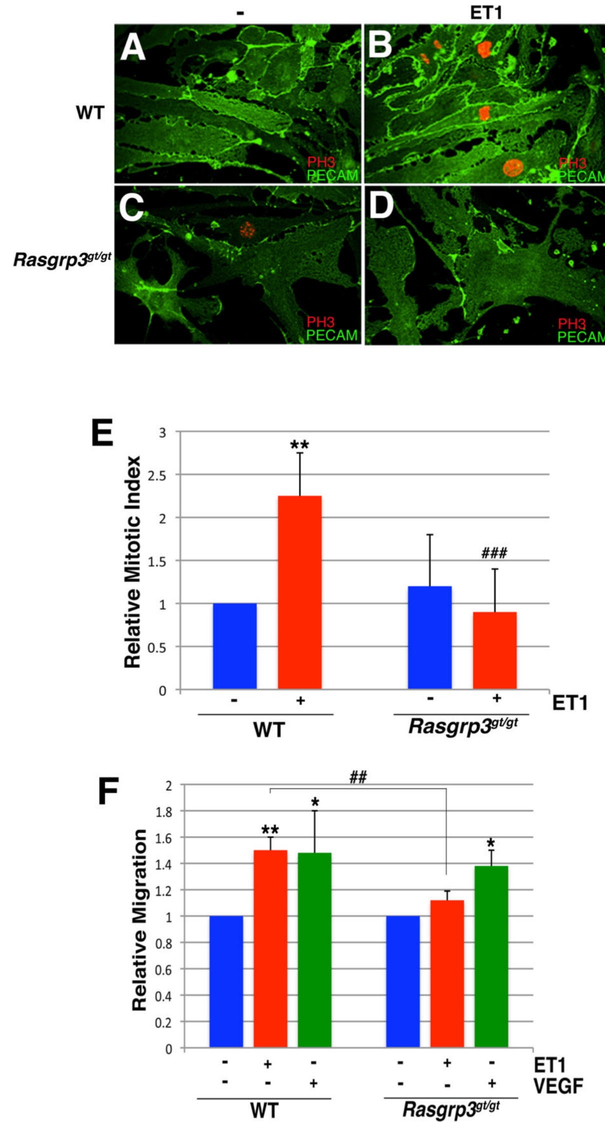

Figure 5. RasGRP3 mediates ET1-induced endothelial cell proliferation and migration

Control (**A, C**) or ET1-treated (**B, D**) WT (**A, B**) or *Rasgrp3*$^{gt/gt}$ (**C, D**) endothelial cells were stained for PECAM (green) and PH3 (red). **(E)** Representative areas were imaged and mitotic cells counted. The relative mitotic index was significantly increased in WT endothelial cells treated with ET1, but was not significantly increased in *Rasgrp3*$^{gt/gt}$ endothelial cells treated with ET1. **, p 0.001 relative to WT untreated; ###, p 0.001 relative to ET1-treated WT endothelial cells. (**F**) Transwell migration assays. WT endothelial cells had significantly increased migration towards both ET1 and VEGF-A compared to control, whereas *Rasgrp3*$^{gt/gt}$ endothelial cells had significantly increased migration towards VEGF-A but not towards ET1. *, p 0.01 relative to untreated; **, p 0.0001 relative to untreated; ##, p 0.0001, *Rasgrp3*$^{gt/gt}$ ET-1 treated vs. WT ET1-treated.

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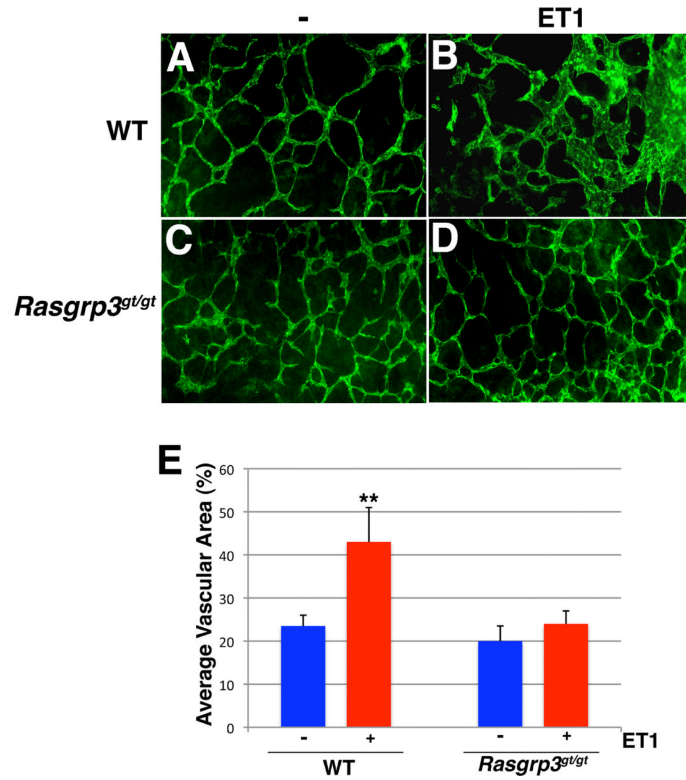

Figure 6. RasGRP3 mediates ET1-induced vessel dysmorphogenesis

ES cell cultures that were WT (**A, B**) or *Rasgrp3*$^{gt/gt}$ (**C, D**) were untreated (**A, C**) or treated with ET1 (**B, D**), then fixed and stained for PECAM (green) on day 8. **(E)** Representative images were quantified for vascular area. WT vessels treated with ET1 had significantly increased vascular area, whereas ET-1 treated *Rasgrp3*$^{gt/gt}$ vessels did not have increased vascular area. **, p 0.01 relative to control.

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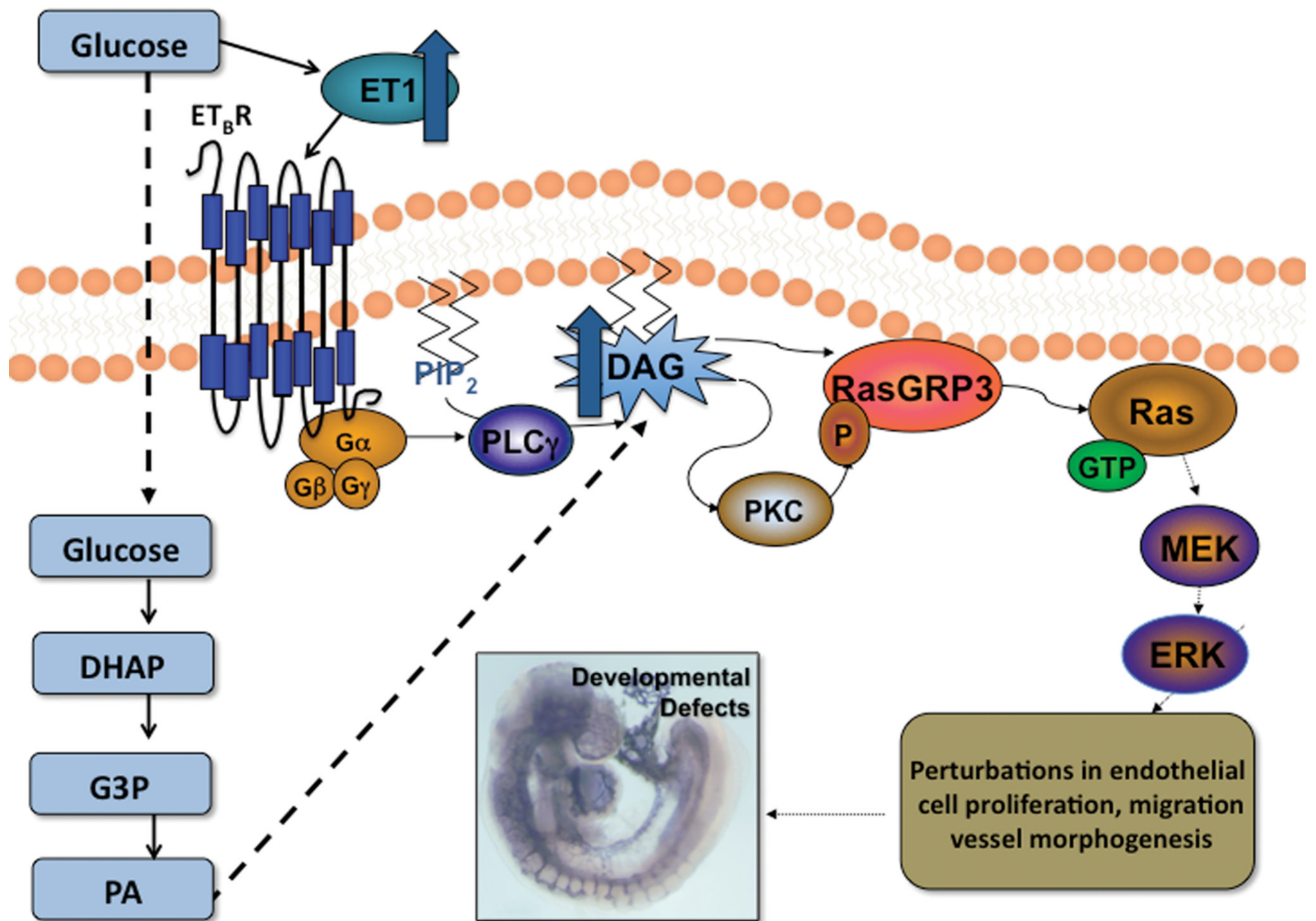

Figure 7. Model of activated RasGRP3 signaling and function in endothelial cells

In this model, maternal diabetic conditions induce elevated DAG production in embryos via aberrant glucose metabolism and via ET1 signaling; endothelial RasGRP3 is over-activated by DAG and PKC phosphorylation to activate Ras (Ras-GTP). Ras signaling through MEK/ERK affects the endothelial actin cytoskeleton and perturbs migration, leading to developmental defects.