THE ROLE OF RASGRP3 IN THE EMBRYONIC RESPONSE TO PHORBOL ESTERS AND DIABETES

Joanna H. Fried

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Approved by:

Advisor: Victoria L. Bautch

Reader: Mark Peifer

Reader: Adrienne Cox

ABSTRACT

Joanna H. Fried: The Role of RasGRP3 in the Embryonic Response to Phorbol Esters and Diabetes

(Under the direction of Victoria L. Bautch)

RasGRP3 is a novel endothelial diacylglycerol (DAG) receptor expressed in developing blood vessels. Because DAG levels are elevated in diabetes, I hypothesized a role for RasGRP3 in the embryonic response to disrupted DAG signaling. Using whole embryo culture, I ascertained differences between wildtype embryos and embryos genetically deleted for RasGRP3 exposed to excess glucose or phorbol 12-myristate 13-acetate (PMA), a compound that mimics the effects of diacylglycerol. Wildtype embryos exhibited numerous vascular and nonvascular defects when cultured with excess glucose and increased severity of defects when cultured with PMA, most notably in the yolk sac vasculature. Deletion of RasGRP3 partially protected embryos from defects experienced by wildtype embryos cultured with PMA or glucose. I also analyzed embryos developing in a maternal diabetic environment induced by streptozotocin injection. In this system, wildtype embryos had defects similar to those seen in embryo culture studies, while embryos deleted for RasGRP3 were partially protected.

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BSA	bovine serum albumin
DAG	diacylglycerol
dL	deciliter (0.1 liter)
dNTPs	deoxynucleoside triphosphates
ES	embryonic stem
gt	gene trap
mg	milligram
μL	microliter
mM	millimolar
nM	nanomolar
PECAM	platelet endothelial cell adhesion molecule
PBS	phosphate-buffered saline
PBT	0.2%BSA, 0.1% Triton X-100 diluted in PBS
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate (a phorbol ester)
RasGRP3	ras guanosine nucleotide releasing protein 3
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VE-PTP	vascular endothelial cell-specific phosphotyrosine phosphatase

CHAPTER 1: INTRODUCTION

1.1. Blood vessel development

Proper development of blood vessels is critical both during embryonic development (to provide nutrients and allow gas exchange in developing organs) and during specific events in adulthood, such as during wound healing and growth of placental vessels during pregnancy. Blood vessels develop via coordination of two processes: vasculogenesis and angiogenesis. Vasculogenesis occurs as blood vessel precursors called angioblasts proliferate, differentiate, and migrate to form primitive blood vessels. Angiogenesis occurs as those primitive vessels are remodeled through further migration and proliferation. Processes such as sprouting of new vessels off pre-existing ones and pruning of existing vessels help to form a branched vascular plexus (for review: Beck and D'Amore 1997). This remodeling process occurs along with recruitment of pericytes and smooth muscle cells to bolster the walls of some vessels. Both vasculogenesis and angiogenesis must occur properly to form a fully functional vascular plexus, and it is the coordination of these processes with other developmental programs that result in temporally and spatially consistent vascular patterning (for review: Hogan and Bautch 2004). Blood vessel development begins around E7.5 in mice and continues throughout gestation. During mid-gestation, it generally progresses in an anterior to posterior fashion.

1.2. Signaling pathways

Many signaling pathways contribute to blood vessel patterning, including the Notch, Ephrin, and TGF-β pathways, as well as a variety of signals controlling cell-cell and cell-matrix interactions (Jain 2003). In our lab, we are primarily interested in the VEGF-A and Ras signaling pathways. We have only recently developed an interest in the Ras signaling pathway, as recent work in the lab has discovered a VEGF-responsive gene involved in the Ras signaling pathway that is expressed in developing blood vessels. A more detailed introduction to these signaling pathways and our interest in them is provided below.

VEGF signaling

The VEGF family of mammalian proteins contains five members, all of which are secreted ligands that bind to tyrosine kinase receptors on their target cells. Family members include VEGF-A, VEGF-B, VEGF-C, VEGF-D, and Placental Growth Factor (PlGF), all of which have roles in development (for review: Yamazaki and Morita 2006). VEGF-A was the first family member discovered and has been the most well-characterized. It has diverse roles in the developing embryo, functioning in the patterning of bone and the nervous system as well as in its well-characterized role as a potent pro-angiogenic factor (for review: Coultas et al. 2005).

VEGF-A (hereafter called VEGF) exists as several isoforms generated by alternative splicing that are secreted from hypoxic cells and differ in their degree of association with the ECM, thereby presumably providing some molecules which can diffuse to more distant targets while others remain near the non-endothelial source

(Ruhrberg et al. 2002). When VEGF reaches its target cell, it binds the high-affinity membrane-bound receptor Flk1 (VEGFR-2 in humans). Flk1 mediates most of the downstream intracellular signaling functions of VEGF, including increased proliferation, migration, and survival of endothelial cells (Ferrara et al. 2003 ; Cross et al. 2003).

In addition to signaling through Flk-1, VEGF also binds the soluble or membrane-bound receptor Flt-1 (VEGFR-1) which acts as a negative regulator of angiogenesis during development (Kearney et al. 2002; Kearney 2004; Tanaka et al. 1997; Shibuya 2001). Mouse-derived ES cells can differentiate *in vitro* to form numerous cell types, including endothelial cells. ES-cell-derived blood vessels mutant for Flt1 experience an overgrowth of endothelial cells (Kearney et al. 2002), making this mutation the equivalent of a VEGF gain of function. A similar phenotype has been observed *in vivo* in embryos with a Flt1 null mutation (Fong et al. 1995; Kearney et al. 2002).

In addition to Flk-1 and Flt-1, VEGF binds Neuropilin-1 (Npn-1) on endothelial cells (Gu et al. 2003). Npn-1 acts as a co-receptor for VEGF, enhancing its signaling through Flk-1 to regulate angiogenesis by stimulating branching and growth of blood vessels (Soker 2002). There are many VEGF-responsive genes expressed during development. One of these is the Ras activator RasGRP3. This was shown by Northern blot analysis of human endothelial cells stimulated with VEGF-A (Roberts et al. 2004). This, as well as work from other labs, provides evidence for a link between the VEGF and Ras signaling pathways during angiogenesis (Berra et al. 2000; Kranenburg et al. 2004).

Ras signaling

The Ras superfamily of proteins consists of more than 20 members that are involved in a wide variety of cellular processes. The members of this superfamily in which we are particularly interested are the small GTPase Ras and its closest relative, Rap1. We are interested in these two proteins because we know they are activated by our protein of interest, RasGRP3 (see below). Ras is localized primarily to the plasma membrane and has a wide variety of downstream effectors, resulting in modulation of gene expression of various targets. One key endpoint of Ras activation in some cell types is the destabilization of cell-cell junctions, which occurs by downstream signaling through the MAPK cascade involving Mek and Erk (Lu et al. 1998).

Rap1 is another small GTPase, but it is localized predominantly in the perinuclear region (specifically, in the endoplasmic reticulum and Golgi apparatus). Rap1 has some of the same downstream effectors as Ras, and early studies suggested that Rap1 functioned to competitively inhibit Ras activity by binding to some of the same downstream effectors, such as Raf1 (Repasky et al. 2004). More recent studies, however, have found numerous independent downstream pathways through which Rap1 may function (Kooistra et al. 2007). One key function of Rap1 is the stabilization of cell-cell junctions (Bos 2005; Knox and Brown 2002), which it accomplishes by regulating cadherin-based cell-cell contacts as well as assembly and disassembly of myosin-II (Jeon et al. 2007). Rap1 is also important in establishing and maintaining cell polarity (Schwamborn and Puschel 2004).

Ras family members exist in an inactive GDP-bound state and are activated by several families of guanine exchange factors (GEFs). A GEF forms a complex with the

Ras family member to cause release of the GDP molecule, allowing binding of GTP to take place, thereby activating the Ras protein (for review: Bourne et al. 1990). The specificity of RasGEFs for the various Ras family members is complicated, with some GEFs activating multiple proteins and some proteins being activated by multiple GEFs (Tian and Feig 2001). Three families of RasGEFs have been identified thus far: Son-of-sevenless (Sos), Ras guanosine nucleotide releasing factors (RasGRFs), and Ras guanosine nucleotide releasing proteins (RasGRPs) (for review: Quilliam et al. 2002). Of these, we are most interested in the RasGRP family because it contains two members expressed in the developing vasculature.

There are four members of the RasGRP family of RasGEFs. These proteins are all similar structurally but are localized to different cell types and have varying specificities for which specific Ras family member they activate. RasGRP3 is of most interest to us, for the reasons detailed below.

1.3. RasGRP3

Our lab became interested in RasGRP3 before I joined, based on its identification through a gene-trap screen looking for novel genes expressed in the developing vasculature. The insertion of the lacZ gene-trap resulted in a null mutation ($RasGRP3^{gt/gt}$), but mutant mice are viable and fertile and are born at expected Mendelian frequencies (Roberts et al. 2004). Although these results showed that RasGRP3 is not required during normal development, my thesis project focused on understanding its potential role in development during situations when normal signaling is disrupted.

RasGRP3 is one of the first non-PKC diacylglycerol (DAG) receptors to be described (Teixeira et al. 2003). Like other RasGRP family members and unlike

members of the other two families of RasGEFs, RasGRP3 is not tethered to a membrane-bound receptor. Instead, it can be found localized subcellularly in the perinuclear region, specifically in the endoplasmic reticulum and Golgi apparatus (Lorenzo et al. 2001). Due to this localization, RasGRP3 is able to activate the specific population of Ras localized to the Golgi, unlike other Ras activators tethered via adaptor proteins to the plasma membrane and restricted to activating the Ras there (Caloca et al. 2003). However, if RasGRP3 becomes localized to the plasma membrane, it can also activate that populations of Ras or Rap localized there (Stope et al. 2004).

RasGRP3 contains several functional motifs that are also common to other RasGRP family members. These motifs include a catalytic GEF domain that is active in removing the GDP molecule from RasGRP3 targets, a pair of calcium-binding E/F hands whose roles are still being elucidated, and also a C1 domain. The C1 domain is essential in that it is responsible for binding DAG to target RasGRP3 to the plasma membrane. This reliance on the second messenger DAG sets RasGRP3 apart from the Sos and GRF families of RasGEFs, and the specificity of its C1 domain differs from that of other GRP family members, some of which bind phospholipids other than DAG and have varying affinities for DAG and phorbol esters (Johnson et al. 2007). Another difference between RasGRP3 and other family members is the existence of a DLC1 domain in RasGRP3, which binds dynein light chain 1 (Okamura et al. 2006). In addition to a requirement for binding DAG, RasGRP3's GEF function seems to hinge on its phosphorylation state, and this phosphorylation is PKC-dependent (Zheng et al. 2005). RasGRP3 has been shown in vitro to activate Ras, Rap1, and R-Ras (Rebhun et al. 2000; Reuther et al. 2002).

As mentioned previously, RasGRP3 is a VEGF responsive gene. It is expressed in endothelial cells and is upregulated in response to a VEGF signal. RasGRP3 is expressed during development in the developing blood vessels and early somites. It is downregulated in adult tissues but is reexpressed in adult blood vessels undergoing physiological or pathological angiogenesis, such as in maternal vessels of the decidua during pregnancy and in the invading vessels during tumorigenesis (Roberts et al. 2004).

Redundancy between other RasGRP family members may explain the lack of phenotype in RasGRP3$^{gt/gt}$ mice, and RasGRP2 is the most likely candidate for redundancy because it is the only other RasGRP family member expressed in an endothelial cell line (D. Roberts, unpublished data). It is also the only other family member that has been reported to activate both Ras and Rap1 (Clyde-Smith et al. 2000).

The results of previous experiments in our lab led us to hypothesize a role for RasGRP3 in development during abnormal signaling events. DAG activity can be mimicked *in vitro* through use of the compound phorbol 12-myristate 13-acetate (PMA), which induces RasGRP3 relocalization to the plasma membrane (Lorenzo et al. 2001). When ES cultures differentiated in vitro are treated with 100nM PMA, they show a phenotype marked by formation of endothelial sheets which looks very similar to the previously mentioned phenotype of Flt1 mutant endothelial cells. RasGRP3$^{gt/gt}$ cells are refractory to this PMA treatment and form a normal vascular plexus when treated with PMA (Roberts et al. 2004). Therefore, even though the viability of RasGRP3$^{gt/gt}$ mice indicates that RasGRP3 is not required under normal physiological conditions, the phenotype seen in this differentiated ES cell experiment suggests it is potentially required to mediate certain abnormal/disease states, most likely when DAG is elevated.

1.4. Diabetes

Diabetes is one disease that has been shown both *in vitro* and *in vivo* to be marked by elevated DAG levels in many cell types (Craven 1989; Verrier et al. 2004). Evidence indicates that these elevated DAG levels result from an increase in *de novo* DAG synthesis during metabolism of the elevated amounts of glucose present in diabetes (Craven 1990). In diabetes, DAG is synthesized *de novo* from products of glycolysis such as dihydroxyacetone phosphate and glycerol 3-phosphate (Lee et al. 1989). Elevated DAG levels highly correlate with many of the complications of diabetes and are accompanied by an increase in activation of PKC. DAG activates PKC by targeting it to the plasma membrane by binding its C1 domain, and activated PKC is involved in activation of numerous signaling pathways which result in many of the vascular complications of diabetes (Koya and King 1998).

The physiological response to elevated blood glucose levels depends on hormone production by the pancreas. The pancreas contains specialized groups of cells called Islets of Langherhans which contain several different cell types grouped around interspersed capillaries. The two pancreatic cell types in these islets that are most relevant to diabetes are the β-cell and the α-cell, each of which is specialized to produce one hormone important in glucose homeostasis. Under normal physiological conditions, a feedback loop exists in the body such that elevated blood glucose levels stimulate the β-cells to increase their production of insulin, while lower blood glucose levels cause the α-cells to increase their production of glucagon. Glucagon stimulates hydrolysis of glycogen in the liver to release more glucose into the bloodstream to bring blood glucose levels back up. Conversely, insulin functions both at the liver to stimulate increased

storage of glucose as glycogen and at a wide assortment of body tissues to stimulate increased uptake of glucose from the blood. Both of these functions of insulin result in blood glucose levels decreasing back to an acceptable level.

Diabetes results when there are problems in this feedback system that allow the blood glucose level to remain consistently higher than normal. There are 2 types of diabetes, both of which result in hyperglycemia but through different mechanisms. Type I diabetes is an autoimmune disease caused by destruction of pancreatic β-cells leading to loss of insulin production. With no insulin production, blood glucose levels climb and tissues are exposed to pathogenic concentrations of glucose. Type II diabetes, on the other hand, is caused by cells developing insulin-resistance. In Type II diabetes, the pancreas still senses hyperglycemia and responds by increasing insulin production, but target tissues have reduced capacity to respond to the insulin signal and fail to filter the glucose from the blood.

Diabetes is a disease marked by problems in practically every system in the body, including numerous occurrences of vascular complications, or vasculopathy. These complications directly correlate with the level of hyperglycemia that exists (Algenstaedt et al. 2003). One example of diabetic vasculopathy in the adult occurs in the retina, where numerous vascular problems occur that are collectively termed diabetic retinopathy. Diabetic retinopathy is marked by increased permeability (leakiness) and occlusion of some vessels, resulting in hypoxia that induces neovascularization, which is growth of some vessels into what are supposed to be avascular areas (for review: Gariano and Gardner 2004; Zhang et al. 2005). These problems will eventually cause blindness, as hemorrhaging and accumulation of scar tissue lead to retinal detachment. Diabetic

retinopathy is one of the leading causes of blindness in the world. Diabetic nephropathy, or kidney disease, is also a vascular problem. Progressive thickening of the glomerulus eventually destroys the kidney's filtering capacity, and leaky vessels allow important components such as serum proteins to pass into the urine (for review: Mogensen 1983). There is also an increased incidence of cardiovascular disease (Haffner et al. 1998), as well as problems such as limb ischemia that often result in amputation of limbs.

In addition to these problems in the adult, there is also a two to four-fold increase in the incidence of birth defects in offspring of diabetic females compared to offspring of nondiabetic mothers (Eriksson 1991). These birth defects include both vascular and nonvascular problems, with cardiovascular complications among the most common defects observed (Becerra 1990). Common nonvascular problems include neural tube closure defects and improper patterning of the body axis. Vascular problems include many defects in tissues such as the yolk sac and placenta that form the interface between mother and fetus and that are key to maintaining nutrient supply between the maternal systems and the developing embryo.

Normal vascular patterning of the murine yolk sac proceeds in a well-characterized fashion. Endothelial precursor cells are visible by E7.0, very shortly after origination of the yolk sac, and by E10.0 a fully functional circulation is present (Auerbach et al. 1996). At E8.5, a plexus of primitive, homogeneous vessels has formed, which is remodeled between E8.5 and E9.5 to form a hierarchial arborized plexus by E9.5 (Wang 1998). This is also the time during which bloodflow is established through the yolk sac vasculature and the embryo becomes dependent on it for survival. Defects in the yolk sac vasculature can therefore result in embryonic defects or fatality. Diabetes-

related defects in the yolk sac vasculature have been observed in yolk sacs of embryos grown in hyperglycemic conditions using whole embryo culture as well as in yolk sacs of embryos from streptozotocin-induced diabetic mothers (Pinter et al. 1999; Pinter et al. 2001). These defects may result from disruption of normal VEGF-A signaling during hyperglycemia (Pinter et al. 2001).

Because DAG is elevated in diabetes and RasGRP3 is activated by DAG, I hypothesized a role for RasGRP3 in mediating the embryonic vascular defects seen in diabetes. By treating embryos with DAG-mimicking PMA in a whole-embryo culture system, I can determine whether RasGRP3 has a role in mediating the embryonic response to this compound. Also, by using the whole-embryo culture system and treating embryos with excess glucose, I can determine whether RasGRP3 mediates the response of embryos to elevated glucose levels. I also used another, more biologically-relevant approach to this question by creating diabetic mice via streptozotocin injection. Streptozotocin has been used for years to generate Type I diabetes in lab animals. By using this system, I can compare effects of diabetes on development of embryos in mice that are wildtype or mutant for RasGRP3. I hope by these experiments to further understand the role of RasGRP3 in development, specifically in systems experiencing abnormal signaling events.

CHAPTER 2: MATERIALS AND METHODS

Rat serum isolation

Rat serum was obtained using 19-gauge needles to collect blood from the aortas of retired male breeder rats deeply anesthetized with ether. Blood was decanted into 15mL conical tubes and immediately centrifuged at speed setting 7 on a tabletop centrifuge at room temperature. Serum was removed from the top layer with a pasteur pipet and collected in a fresh tube on ice. The fibrin clot in the original tube was squeezed with forceps and then centrifuged again. Serum was removed with a pipet after each centrifuge step until no additional serum was released from the clot. Serum was pooled into groups A, B, and C based on its quality, with A being the highest-quality, hemolysis-free serum. Serum from batch A was used for all embryo culture experiments. Serum was heat-inactivated for 30 minutes at 56°C. Serum was then stored at -80°C until immediately before use, when it was thawed at room temperature and had 5% CO_2 bubbled through it for two minutes to eliminate residual ether.

Whole embryo culture

Embryos were dissected into M2 media from maternal decidua with their yolk sacs intact. Dissections were performed on either E7.5 or E8.5 (based on counting midday of the day a plug was observed as E0.5). Embryos were cultured for 24 hours in 25mL 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-hour intervals with a gas mixture containing 20% O_2 and 5% CO_2 and the tubes were sealed with vacuum grease. Some embryos started in culture at E7.5 were treated with 50nM PMA (phorbol 12-myristate 13-acetate), while some embryos started in culture at E8.5 were treated with 20mM glucose. After 24 hours, embryos were dissected away from the yolk sac in PBS. Wholemount pictures were taken before and after dissection, after which embryos were fixed for staining.

Genotyping of embryos from RasGRP3$^{gt/+}$ x RasGRP3$^{gt/+}$ crosses

Embryos from RasGRP3 intercross litters were genotyped using a fragment of yolk sac removed during the final dissection. PBS was removed from the tissue and the tissue was stored at -20°C in 1.5mL tubes until use. DNA was extracted from yolk sacs by incubation for one hour at 95°C in 30μL of 25mM NaOH + 0.2mM EDTA, followed by neutralization with 30μL of 40mM TrisHCl, pH 5. Two PCR amplification reactions were performed for each sample: a RasGRP3 reaction which ran for 30 cycles and a LacZ reaction which ran for 36 cycles. Each LacZ reaction contained 5 μL DNA, 11.625 μL dH_2O, 2.5 μL 10X Qiagen PCR buffer with dye, 0.2 μL 25mM dNTPs, 0.25 μL each forward and reverse primers, 5 μL Q buffer, and 0.175 μL Taq polymerase. Each RasGRP3 reaction contained 5 μL DNA, 11.725 μL dH_2O, 2.5 μL 10X Qiagen PCR buffer with dye, 0.2 μL 25mM dNTPs, 0.2 μL each forward and reverse primers, 5 μL Q buffer, and 0.175 μL Taq polymerase The primers for the RasGRP3 reaction were forward 5'-AGAGAACCACTGCCTCGTAC-3' and reverse 5'-GTGTTGCCGCTTTCCCGAGC-3'. The primers for the LacZ reaction were forward 5'-

TTGAAAATGGTCTGCTGCTG-3' and reverse 5'-TTGGCTTCATCCACCACATA-3'. Products were analyzed on a 3% agarose/TBE gel with ethidium bromide.

Wholemount PECAM staining of embryos and yolk sacs

Embryos were dissected into ice-cold PBS and then fixed in 4%PFA/PBS overnight at 4°C, with one embryo or yolk sac per well of a 48-well plate. All wash steps used a minimum 200µL of solution per well. Embryos were washed 15 minutes in PBS, then dehydrated through a methanol:PBS series (15 minutes each, same methanol:PBS series described above). Endogenous peroxidase activity was blocked by incubating 4 hours at RT with 5%H_2O_2 in methanol. Embryos were stored overnight in 100% methanol at -20°C, then rehydrated through a methanol/PBS series, blocked 2x 1 hour in PBT (0.2%BSA, 0.1% Triton X-100 in PBS), and incubated overnight at 4°C in 1:200 rat anti-mouse CD-31 (PECAM) primary antibody (BD Pharmingen 553370) in PBT. Embryos were rinsed 5x 1hour 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 5x 1hour in PBT, then developed for 20 min. at RT in DAB solution (Vector Laboratories, cat. #SK-4100). After developing, embryos were rinsed 2x 5 minutes in PBT, 2x 5 minutes in PBS, and fixed overnight at 4°C in 2%PFA/0.1%glutaraldehyde in PBS. Embryos were rinsed 3x 5 minutes in PBS, then stored in PBS at 4°C.

Xgal staining for β–galactosidase expression

Embryos were dissected into PBS, then washed 1x 5 minutes in 0.1M phosphate buffer (0.1M sodium phosphate monobasic, 0.1M sodium phosphate dibasic). Embryos were fixed (15 minutes for E7.5 or 8.5 embryos, 30 minutes for E9.5 or 10.5 embryos) in gluteraldehyde fixative made fresh before use. Embryos were then washed 3x 15 minutes in Xgal wash buffer at room temperature, then incubated overnight at 37°C in Xgal stain (made fresh every two weeks). The following morning, embryos were washed 1x 15 minutes in Xgal wash buffer, then fixed overnight at 4°C in gluteraldehyde fixative. The following morning, embryos were washed 1x 5 minutes in PBS, then underwent paraffin embedding and sectioning as described above.

Paraffin embedding and sectioning of embryos

After PECAM antibody-staining or Xgal staining, embryos were dehydrated through a PBS:methanol series (100% PBS, 75% PBS:25% methanol, 50% PBS:50% methanol, 25% PBS:75% methanol, then 100% methanol for 10 minutes each), then were transferred to glass vials and incubated 2x 10 minutes in 100% ethanol. Embryos were cleared 3x 5 minutes in HistoClear, then changed to 100% paraffin for 1 hour. Embryos were then incubated overnight at 58°C in fresh paraffin. Paraffin was changed out until all residual HistoClear had been removed, at which time the embryos were embedded in disposable plastic embedding molds and left to harden overnight. Twelve μM sections were taken and floated on distilled water on slides and left to dry overnight. Sections were cleared for 7 minutes in HistoClear and then rehydrated through an ethanol:PBS series (100% ethanol, 75% ethanol:25% PBS, 50% ethanol:50% PBS, 25% ethanol:75%

PBS, 100% PBS, 100% PBS, all for 2 minutes each). Rehydrated slides were dried for 2 minutes, after which several drops of GlycerGel Mounting Media were placed on the slide and a coverslip was floated on top and allowed to set overnight.

Induction of diabetes via streptozotocin injection

Streptozotocin (stored at -20˚C) was dissolved to a final concentration of 7.5mg/mL in 0.1M Na-Citrate buffer (pH 4.5) immediately prior to injection. Female mice (ideally 6-8 weeks of age and weighing 20-25g) were injected intraperitoneally with a dose of 50mg STZ per kg mouse (on average, a volume of 150-200μL per mouse) for 5 consecutive days. Injections were given after noon so that mice would have fasted several hours pre-injection (because they are nocturnal). Blood glucose levels of mice were monitored using a OneTouch Ultra device to read glucose levels of blood acquired from a nick in the distal portion of the tail. Mice were considered diabetic with blood glucose levels over 250mg/dL. Most mice become diabetic by 2 to 3 weeks after the last injection and remained diabetic long-term. Once mice were diabetic, they were mated and embryos were dissected out at E9.5.

CHAPTER 3: RESULTS

3.1. Effects of RasGRP3 on the embryonic response to phorbol ester

As mentioned previously, RasGRP3 is required in differentiated ES cells for a response to treatment with the phorbol ester PMA. Wildtype ES cells form endothelial sheets in response to PMA treatment while RasGRP3$^{gt/gt}$ ES cells are refractory to this response (Roberts et al. 2004). I wanted to test the hypothesis that RasGRP3 is also required *in vivo* for the embryonic endothelial response to PMA.

I tested this hypothesis using whole embryo culture to grow E7.5 mouse embryos for 24 hours either with or without PMA. I chose this stage of development because the embryo is not yet fully dependent on the yolk sac for survival at E7.5. PMA is known to disrupt development of the yolk sac (Chen 1994), making it imperative that these experiments be performed prior to the stage at which dependence of the embryo on the yolk sac makes any effects of PMA treatment lethal to the embryo. My initial experiments were performed using 100nM PMA. This concentration was chosen based on a dosage curve previously performed showing that the endothelial response to PMA occurred over doses ranging from 10nM to 1000nM (D. Roberts, unpublished results). Embryos in these initial experiments experienced such severe defects that I reduced the PMA concentration to 50nM in subsequent experiments.

My first experiments were performed separately on RasGRP3$^{gt/gt}$ embryos or wildtype embryos. These wildtype embryos were from a strain of mice that express β-galactosidase through a gene trap knockin at the PECAM locus. PECAM (platelet

endothelial cell adhesion molecule) is expressed primarily in endothelial cells, so I used Xgal staining to visualize the developing blood vessels in these embryos. These experiments showed that wildtype embryos exhibited severe defects when treated with 100nM PMA, while RasGRP3$^{gt/gt}$ embryos appeared indistinguishable from controls (Figure 1A-D). As expected based on the gross defects seen in wildtype embryos treated with PMA, quantitation of general health characteristics of embryos in these experiments confirmed that wildtype embryos treated with 100nM PMA were unhealthy compared to control wildtype embryos. Wildtype embryos treated with 100nM PMA had cardiovascular problems such as lack of heartbeat as well as nonvascular problems including failure of somites to develop. RasGRP3$^{gt/gt}$ embryos treated with 100nM PMA were as healthy as their control counterparts based on these marks of general health (Table 1). I also examined the yolk sacs of these embryos, and I noticed severe defects in the yolk sac vasculature of wildtype embryos cultured with 100nM PMA. The vessels appeared abnormally large and were very irregularly-shaped compared to controls (Figure 1E, F). In contrast, RasGRP3$^{gt/gt}$ yolk sacs had a more moderate phenotype. While the vessels in RasGRP3$^{gt/gt}$ yolk sacs appeared larger and more irregular than yolk sacs cultured without PMA, the severity of the phenotype was much reduced compared to that of the wildtype embryos (Figure 1G).

While these results were interesting, it was clear that I needed to reduce the PMA concentration. Culture with 100nM PMA had such drastic and detrimental effects on wildtype embryo development that it was impossible to determine whether any vascular-specific effects had taken place in the embryo. By reducing the PMA concentration to 50nM, I hoped that embryos would undergo more normal development and that I would

be able to see vascular-specific defects rather than gross morphological defects. Wildtype embryos cultured with 50nM PMA did, in fact, develop much more normally than they did when treated with 100nM PMA, although development was still clearly affected by the PMA (Figure 1H).

In addition to reducing the PMA concentration to clarify the results, I also wanted to perform a better-controlled experiment. I accomplished this first by switching my staining methods from Xgal staining to PECAM antibody staining to eliminate any differences based on the different reporter strains of mice. Another advantage of using PECAM antibody staining was that intersomitic vessels could be seen in RasGRP3$^{gt/gt}$ mice, which could not be seen in Xgal-stained embryos due to transient somitic expression of RasGRP3 during development. Additionally, I performed experiments on RasGRP3 intercross litters to have internal controls. By crossing two RasGRP3$^{gt/+}$ mice, I had RasGRP3$^{gt/gt}$, RasGRP3$^{gt/+}$, and RasGRP3$^{+/+}$ (wildtype) embryos all included in the same experiment. While these embryos were still not genetically identical, there was a much higher degree of genetic similarity between these siblings than there was between the random and highly-varied genetic backgrounds of the embryos I had used in my initial experiments.

Culturing embryos from RasGRP3 intercross litters with 50nM PMA confirmed the results of my preliminary experiments. Differences between the genotypes were striking immediately after the conclusion of their 24 hours in culture. Some of these embryos that had been treated with 50nM PMA appeared indistinguishable from embryos cultured without PMA (Figure 2A,C,I), while other embryos treated with 50nM PMA exhibited a severe phenotype marked by a puckered or otherwise irregularly-shaped yolk

sac and by abnormal interconnections between the yolk sac and the amnion (Figure 2E). There was also a phenotype intermediate between these two in which the yolk sac's shape was often slightly modified from its normal round appearance and some interconnections between the amnion and yolk sac were visible (Figure 2G).

Based on my initial experiments using 100nM PMA, I hypothesized that the most severe defects were experienced by wildtype embryos and that the embryos indistinguishable from the controls were RasGRP3$^{gt/gt}$ embryos. After characterizing the phenotypes, I determined the embryos' genotypes by extracting DNA from a fragment of each yolk sac. PCR amplification and analysis of these samples confirmed my hypothesis: RasGRP3$^{gt/gt}$ embryos appeared normal when cultured with 50nM PMA, and wildtype embryos had the most severe defects. Interestingly, I found that RasGRP3$^{gt/+}$ embryos experienced an intermediate response to culture with 50nM PMA. Most of these heterozygous embryos had phenotypes that, while abnormal, were not as severe as those seen in wildtype embryos. Some heterozygous embryos appeared completely normal when examined immediately after the 24 hour culture period ended.

After examining the general morphology of these embryos and their yolk sacs, I dissected the embryo away from the yolk sac and performed PECAM antibody staining to analyze the vascular patterning of these embryos. These results mirrored those described above for general morphology. When matched to their genotype, I determined that RasGRP3$^{gt/gt}$ embryos treated with PMA had vessel patterns most similar to the control embryos. They had a vessel plexus forming in the head and also had some intersomitic vessels (Figure 2B,D). Wildtype embryos treated with PMA had the most severe phenotype. They were less developed than the other genotypes even though they

were littermates, and no distinct blood vessel staining was visible (Figure 2F). RasGRP3$^{gt/+}$ embryos again displayed a range of phenotypes: some appeared normal, with a developing head plexus and intersomitic vessels (Figure 2J) while others were smaller and less-developed with no clear vessel staining (Figure 2H). This range of phenotypes in RasGRP3$^{gt/+}$ embryos correlated with the state of abnormality seen in their yolk sacs, with the most abnormal embryos growing in the most abnormal yolk sacs. Table 2 summarizes the general health as well as the specific vascular defects seen in the different genotypes.

Based on these results, I concluded that RasGRP3 is required to mediate the embryonic response to PMA. My RasGRP3 intercross embryo culture experiments provide strong evidence that RasGRP3 is required for embryos to experience the full range of adverse effects from prolonged exposure to phorbol ester. Furthermore, based on the severity of phenotype seen in the yolk sac and amnion, it is possible that defects seen in the embryo proper are secondary to disruptions in development of the yolk sac and connections to the mother. The mechanism for this response is still under investigation. Ongoing experiments seek to further characterize the specific defects in the yolk sac and determine the extent of the yolk sac's role in the response to PMA.

3.2. Effects of RasGRP3 on the embryonic response to elevated glucose levels *in vitro*

Based on results suggesting that RasGRP3 is required for the embryonic response to PMA, I next wanted to determine whether RasGRP3 is also required for embryos to respond to treatment with elevated glucose levels. As previously mentioned, the blood glucose level is elevated in diabetic individuals, and metabolism of this excess glucose

results in higher exposure of cells to DAG. I hypothesized that treatment of embryos with PMA mimicked elevated DAG exposure, so I asked whether embryos incubated with an elevated concentration of glucose also exhibited defects. To test this hypothesis, I performed embryo culture of E8.5 embryos for 24 hours, with or without addition of 20mM glucose. Use of E8.5 embryos and the concentration 20mM glucose were chosen based previous work characterizing defects in wildtype embryos exposed to elevated glucose in a whole embryo culture system during this time period (Pinter et al. 1999).

Results of these preliminary experiments suggest that RasGRP3 is also required for the embryonic response to elevated glucose levels. Wildtype embryos cultured with glucose have defects in the vascular plexus of the head as well as many nonvascular defects such as open neural tubes. RasGRP3$^{gt/gt}$ embryos have no obvious vascular or nonvascular defects when cultured with glucose (Figure 3). It is possible that defects in the wildtype embryos are secondary to yolk sac defects, but further analysis and more experiments would be required to determine this, as yolk sacs were not analyzed in these experiments. This was because the yolk sac phenotype observed in embryo culture experiments with PMA had not yet been observed when these embryo culture experiments with glucose were performed. However, potential defects in the yolk sac of these embryos are most likely not as severe as those seen in wildtype embryos cultured with PMA, because embryos cultured with glucose are still able to establish blood flow in the yolk sac vasculature. In fact, both wildtype and RasGRP3$^{gt/gt}$ embryos cultured with glucose are as healthy as their control counterparts in terms of establishing heartbeat, yolk sac bloodflow, and somite development (Table 3). The maintenance of general health in these embryos even when cultured with glucose suggests that the defects I saw

are more likely to be specifically vascular rather than secondary effects of a nonvascular problem.

3.3. Effects of RasGRP3 on the embryonic response to streptozotocin-induced diabetes

Based on my preliminary experiments with embryos exposed to elevated glucose and on my striking results with RasGRP3 intercross litters of embryos exposed to PMA, I next wanted to see if there was a difference in the way embryos respond *in vivo* to the more complex system of growth in a diabetic maternal environment. I injected female mice with streptozotocin (STZ), which results in destruction of the insulin-producing β–cells of the pancreas to create the same type of situation experienced in type I diabetes. Once these mice were diabetic, I mated them and observed what effects this maternal diabetic environment had on the development of embryos. Embryos would presumably respond to the elevated levels of glucose circulating in the maternal bloodstream by elevating DAG levels. Previous work has shown that approximately 40% of wildtype embryos grown in a streptozotocin-induced diabetic environment exhibit vascular or nonvascular defects (Pinter et al. 1999). Additionally, these embryonic defects have been found to occur in conjunction with defects in the yolk sac vasculature (Pinter et al. 1986). I wanted to know if RasGRP3 must be present in the embryo in order for these defects to occur.

The first embryos from diabetic mothers that I analyzed were wildtype or RasGRP3$^{gt/gt}$ embryos from a mother of the same genotype. These mice were on a mixed genetic background. There was a significantly higher incidence of both vascular and

nonvascular defects in wildtype embryos compared to RasGRP3$^{gt/gt}$ embryos from diabetic mothers (Table 4). Defects seen in wildtype embryos included nonvascular defects such as an improperly patterned body axis or failure of the neural tube to close (Figure 4 A,B,D). Vascular defects such as crooked, thickened, or truncated intersomitic vessels and reduced small vessels in the vascular plexus of the head were also seen (Figure 4A-D). The yolk sac vasculature in these wildtype embryos also had visible defects. A vascular plexus formed in these yolk sacs, but it was not organized as the arborized, hierarchial vascular structure seen in normally-developed yolk sacs (Figure 4H,I). The majority of RasGRP3$^{gt/gt}$ embryos observed developed normally and formed a well-developed head plexus and straight, narrow, evenly-spaced intersomitic vessels (Figure 4E,F), but among those that did have defects, the severity of nonvascular defects was much reduced compared to wildtype. Nonvascular defects in RasGRP3$^{gt/gt}$ embryos were limited to neural tube closure defects, and the only vascular defects observed in these embryos were in the small vessels of the head plexus (Figure 4G). Additionally, all RasGRP3$^{gt/gt}$ yolk sacs showed normal organization of the vascular plexus, with well-defined arborized vessels (Figure 4J). These results showed that when developing in a diabetic maternal environment, RasGRP3$^{gt/gt}$ embryos had a lower incidence of both vascular and nonvascular defects compared to wildtype embryos. (Table 4). When these results were compared to those from the experiments culturing embryos ex vivo with elevated glucose, I found that the incidence of defects was very similar between the two experimental systems. In both cases, wildtype embryos have a higher prevalence of defects in all categories than do RasGRP3$^{gt/gt}$ embryos (Table 5).

These results were striking, but with the genetic variation between the wildtype and RasGRP3$^{gt/gt}$ mice, it was clear that better-controlled experiments were needed to confirm these results. My first attempt to better control these experiments was to create diabetic RasGRP3$^{gt/+}$ female mice and perform intercrosses so that, as in the embryo culture experiments with PMA, I would have RasGRP3$^{gt/gt}$, RasGRP3$^{gt/+}$, and RasGRP3$^{+/+}$ (wildtype) embryos all in the same litter. Genetic variability still exists between the siblings, but they are much more comparable genetically than were the varied genetic backgrounds of the mice used in the original group of STZ-induced diabetic mice. However, I found no predictable pattern to the responses of the different embryonic genotypes to the maternal diabetes. Moreover, none of the embryos from these litters exhibited the severity of phenotype I had seen in the initial experiments (data not shown). Several embryos had neural tube closure defects, but none had the severe body axis defects seen in many wildtype embryos from the original experiment (data not shown). I hypothesized that perhaps the maternal genotype (RasGRP3$^{gt/+}$ in this experiment, RasGRP3$^{gt/gt}$ or RasGRP3$^{+/+}$ previously) affected the embryonic response to a diabetic environment.

To eliminate the variable of maternal genotype, I created diabetic mice on an inbred background, using wildtype C57BL/6J (B6) mice as well as RasGRP3$^{gt/gt}$ mice that had been crossed into the B6 background for 6 generations (N6). These female mice did not show the same severity of response to the STZ treatment as the original outbred mice did; on average the B6 mice became more mildly diabetic than did the outbred mice. As could perhaps be expected of embryos developing in a less hyperglycemic maternal environment, wildtype embryos from these more moderately diabetic B6 mice

had less severe phenotypes than did the wildtype embryos from the more severely diabetic outbred mice. However, significantly fewer defects in RasGRP3$^{gt/gt}$ embryos were scored on the B6 background, regardless of the mother's blood glucose level (Figure 5A,B). Vascular phenotypes of embryos from moderately diabetic wildtype B6 mice were generally limited to minor defects, most noticeable in the intersomitic vessels which could be crooked, thickened, or growing in aberrant locations (Figure 5C,D). RasGRP3$^{gt/gt}$ B6 embryos had less defects, both vascular and nonvascular, than wildtype B6 embryos (Table 6).

Unfortunately, most of the STZ-injected B6 mice that had blood glucose levels as high as those of the outbred mice had severe reproductive problems, and they either did not mate or did not carry the pregnancy successfully to mid-gestation. However, one litter of wildtype B6 embryos were successfully dissected from a mother with a blood glucose level of 513 mg/dL (in the range of those in the initial outbred mouse experiment). Embryos from this litter had a high incidence of neural tube closure and body axis defects, similar to the initial results (Figure 5E-G). These results suggest that RasGRP3 is, in fact, required for the embryonic response to maternal diabetes.

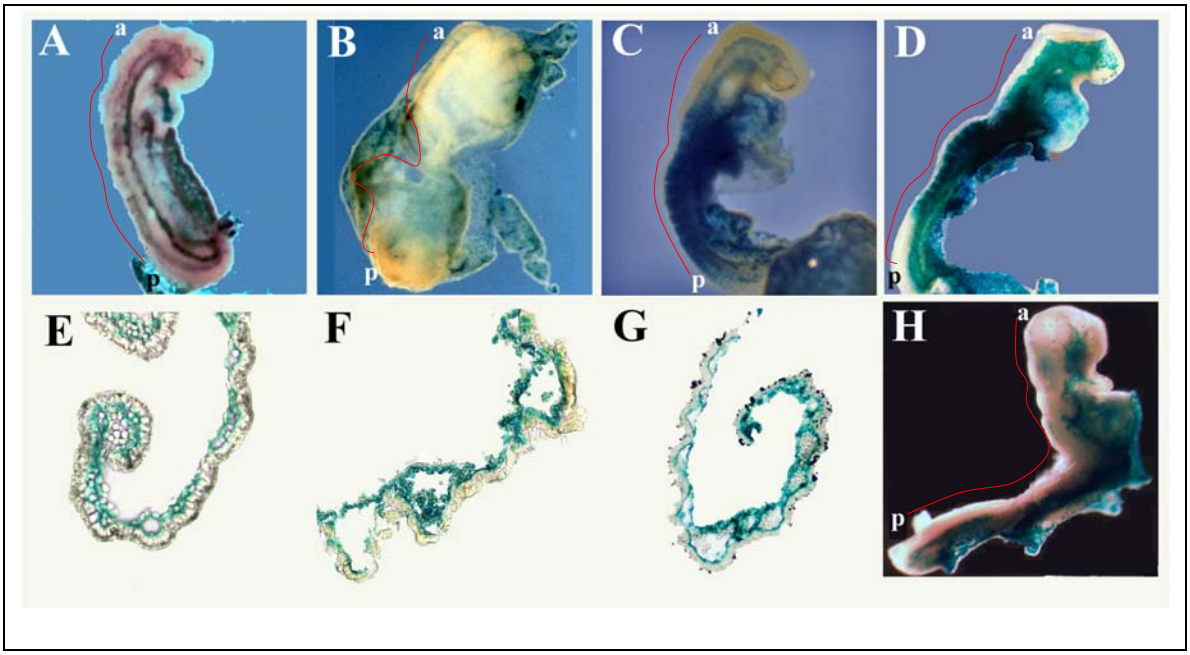

Figure 1: RasGRP3$^{gt/gt}$ embryos are refractory to defects induced by PMA. (A, B, H) Embryos at E8.5 with lacZ inserted into the PECAM locus (wildtype) were cultured 24 hours without PMA (A), with 100nM PMA (B), or with 50nM PMA (H) and stained for β–gal expression. (C and D) Embryos at E8.5 with lacZ inserted into the RasGRP3 locus (RasGRP3$^{gt/gt}$) were cultured 24 hours without (C) or with (D) 100nM PMA and stained for β–gal expression. (E-G) Cross-sections through E8.5 yolk sacs from wildtype embryos cultured without (E) or with (F) 100nM PMA and from (G) RasGRP3$^{gt/gt}$ embryos cultured with 100nM PMA. (A-D,H) a = anterior, p = posterior, red line = body axis).

Genotype	Treatment	Heartbeat	Yolk sac bloodflow	Average number of somites
$PECAM^{lacZ/+}$	Control	100% (5/5)	100% (5/5)	5.2
$PECAM^{lacZ/+}$	100nM PMA	36% (5/14)	0% (0/14)	0.7
$RasGRP3^{gt/gt}$	Control	67% (4/6)	50% (3/6)	9.1
$RasGRP3^{gt/gt}$	100nM PMA	83% (5/6)	17% (1/6)	9.3

Table 1: General health of $PECAM^{lacZ/+}$ and $RasGRP3^{gt/gt}$ embryos after 24 hours in embryo culture with or without 100nM PMA.

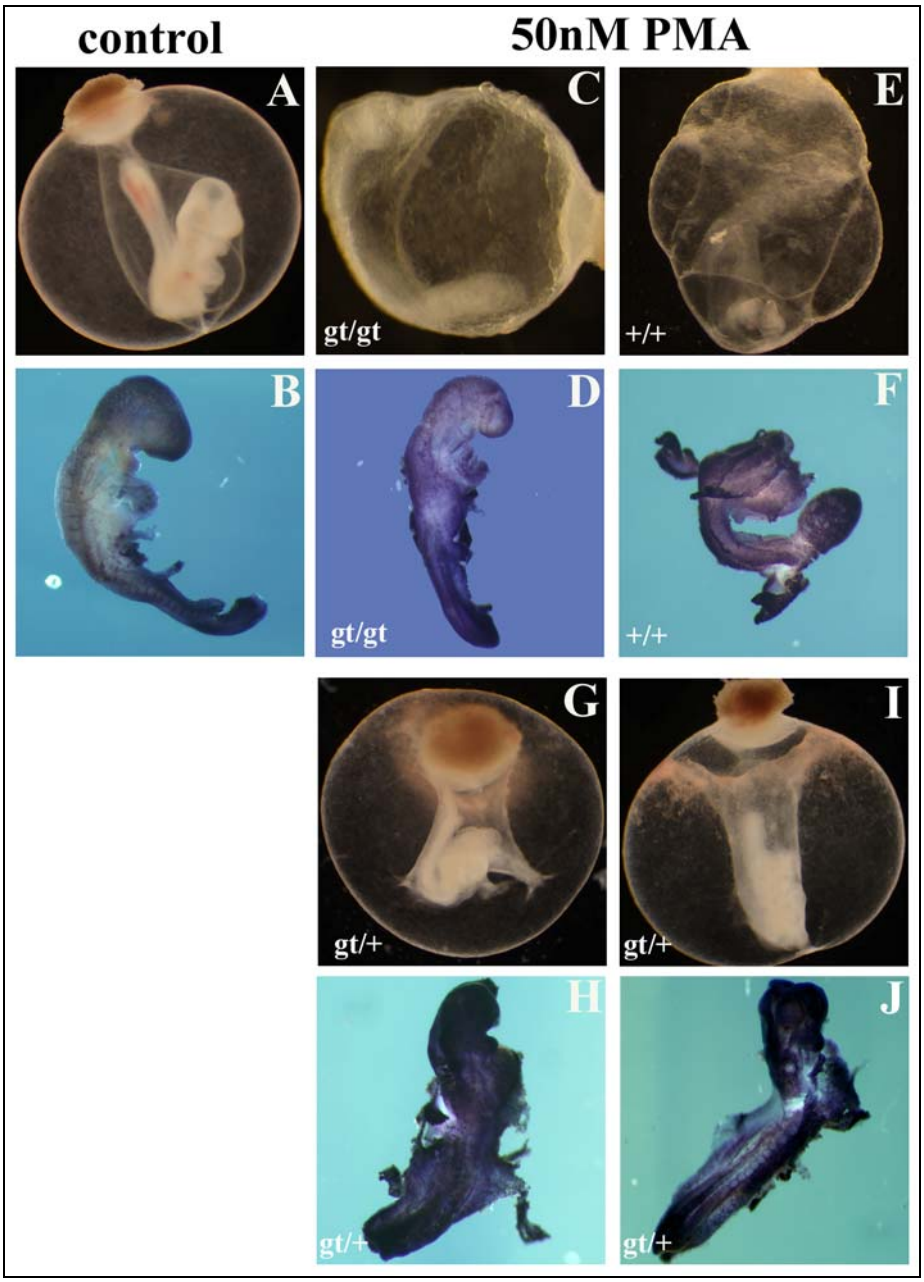

Figure 2: In RasGRP3 intercross litters, RasGRP3$^{gt/gt}$ yolk sac, amnion, and embryo morphology are partially refractory to defects induced by culture with PMA. Embryos growing in the most severely-affected yolk sacs exhibit the most severe embryonic vascular and morphological defects. (A,B) control embryo cultured without PMA, (C-J) embryos cultured 24 hours with 50nM PMA: (C,D) RasGRP3$^{gt/gt}$, (E,F) RasGRP3$^{+/+}$(wildtype), (G-J) RasGRP3$^{gt/+}$. (A,C,E,G,I) embryo in intact yolk sac at termination of 24-hour culture period. (B,D,F,H,J) PECAM-stained embryos. In each set of images, the embryo seen in the upper panel still in its yolk sac is the same embryo seen dissected out and PECAM-stained in the lower panel.

Genotype	Treatment	Heartbeat	Yolk sac bloodflow	Lumpy, abnormal yolk sac	Connections between amnion and yolk sac	Abnormal heartbeat (ie: yolk sac beats w/ heart)	Intersomitic vessels absent or abnormal	Head plexus absent or abnormal	Abnormal body axis	Average number of somites
Wildtype	Control	100% (2/2)	100% (2/2)	0% (0/2)	0% (0/2)	0% (0/2)	n.d.*	n.d.*	0% (0/2)	21
RasGRP3$^{gt/+}$	Control	100% (7/7)	57% (4/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)	9.1
RasGRP3$^{gt/gt}$	Control	100% (2/2)	100% (2/2)	50% (1/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)	19.5
Wildtype	50nM PMA	80% (4/5)	0% (0/5)	80% (4/5)	100% (5/5)	20% (1/5)	100% (4/4)	100% (4/4)	100% (4/4)	4.4
RasGRP3$^{gt/+}$	50nM PMA	87% (13/15)	0% (0/15)	13% (2/15)	73% (11/15)	20% (3/15)	50% (6/12)	83% (10/12)	47% (7/15)	9.1
RasGRP3$^{gt/gt}$	50nM PMA	89% (8/9)	0% (0/9)	11% (1/9)	33% (3/9)	0% (0/9)	57% (4/7)	57% (4/7)	11% (1/9)	6.7

Table 2: Wildtype embryos treated with PMA experience more severe defects than RasGRP3$^{gt/+}$ or RasGRP3$^{gt/gt}$ embryos, although markers of general health are comparable between the different genotypes.

*: Embryos were not PECAM-stained so these traits were not observed.

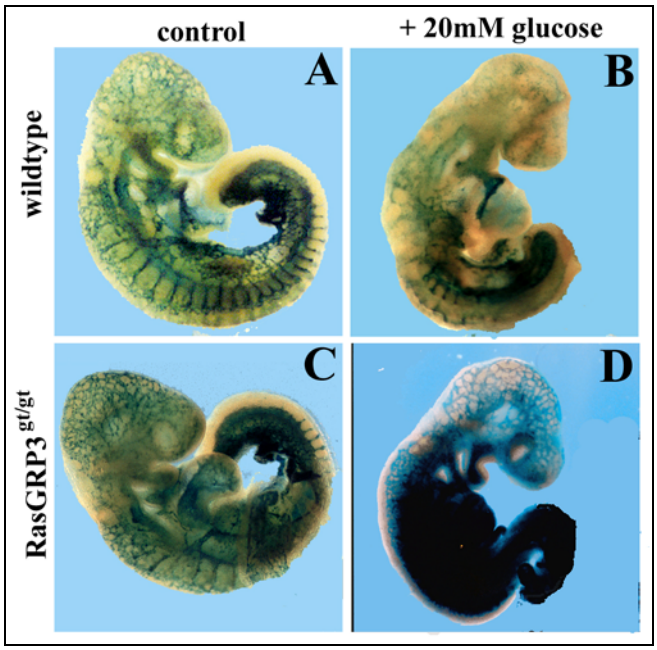

Figure 3: RasGRP3$^{gt/gt}$ embryos are refractory to defects induced by treatment with 20mM glucose. (A and B) E9.5 embryos with lacZ inserted into the PECAM locus, cultured without (A) and with (B) 20mM glucose and stained for β–gal expression. (C and D) E9.5 embryos with lacZ inserted into the RasGRP3 locus (RasGRP3$^{gt/gt}$) cultured without (C) and with (D) 20mM glucose and stained for β–gal expression.

Genotype	Treatment	Heartbeat	Blood flow in yolk sac	Closed neural tube	Anterior limb buds	Average somite number
$PECAM^{lacZ/+}$	Control	100% (15/15)	93% (14/15)	73% (11/15)	93% (14/15)	20.6
$PECAM^{lacZ/+}$	20mM glucose	100% (15/15)	100% (15/15)	60% (9/15)	80% (12/15)	19.2
$RasGRP3^{gt/gt}$	Control	80% (8/10)	60% (6/10)	60% (6/10)	90% (9/10)	17.8
$RASGRP3^{gt/gt}$	20mM glucose	85% (17/20)	60% (12/20)	65% (13/20)	80% (16/20)	18.8

Table 3: General health of $PECAM^{lacZ/+}$ (wildtype) and $RasGRP3^{gt/gt}$ embryos is not impacted by 24 hours in culture with or without 20mM glucose.

Genotype	Environment	Head plexus defects (no head deformity)	Head plexus defects (with head deformity)	Inter-somitic vessel defects	Non-vascular deformities	Average number of somites
Wildtype	normoglycemic mother	0% (0/9)	0% (0/9)	0% (0/9)	11% (1/9)	22.4
Wildtype	diabetic mother	25% (9/36)	33% (12/36)	81% (29/36)	44% (16/36)	21.3
RasGRP3$^{gt/gt}$	normoglycemic mother	8% (2/25)	4% (1/25)	8% (2/25)	4% (1/25)	22.9
RasGRP3$^{gt/gt}$	diabetic mother	8% (1/12)	0% (0/12)	0% (0/12)	8% (1/12)	27.4

Table 4: Comparison of E9.5 PECAM antibody-stained wildtype and RasGRP3$^{gt/gt}$ embryos from normoglycemic or STZ-induced diabetic mothers on outbred backgrounds.

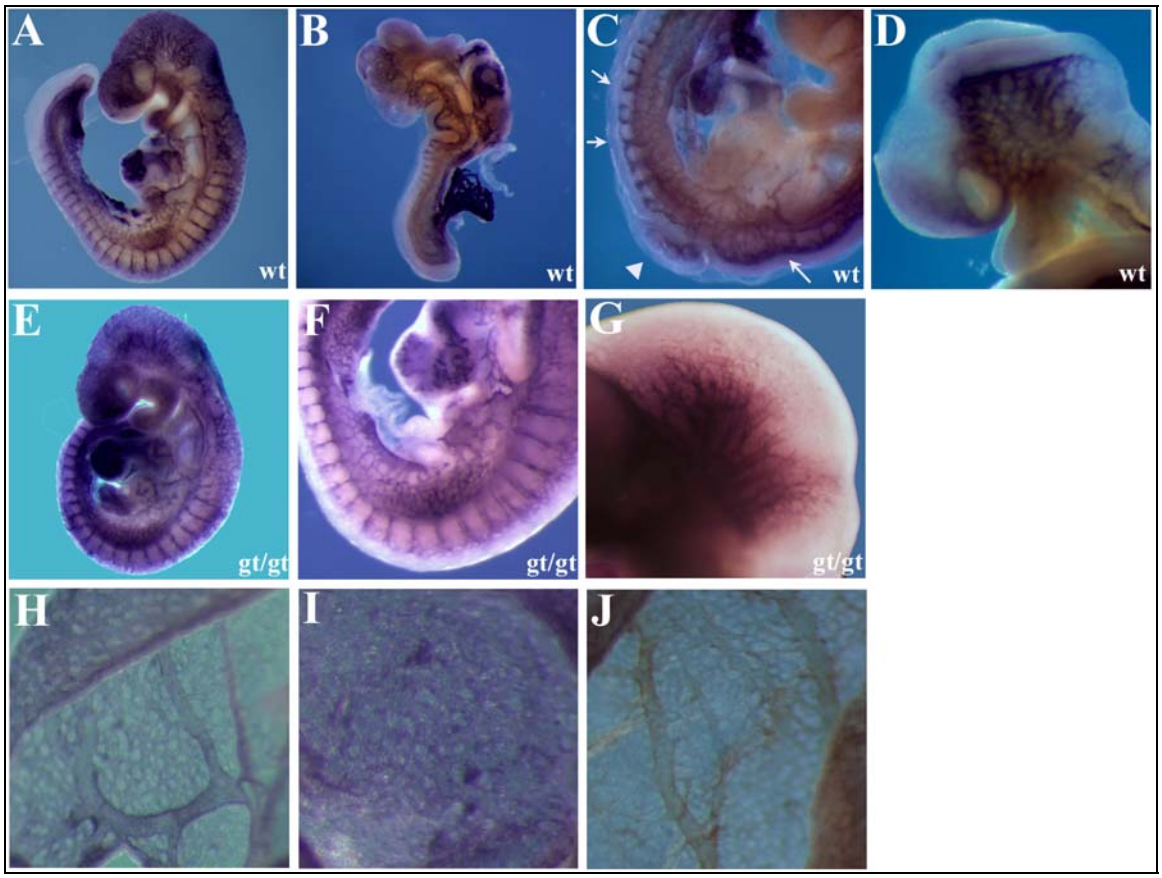

Figure 4: RasGRP3$^{gt/gt}$ embryos are partially protected from defects experienced by wildtype embryos growing in a diabetic mother on outbred backgrounds. (A-D) E9.5 wildtype (mixed genetic background) embryos dissected from mothers with blood glucose levels in excess of 600 mg/dL (33mM) exhibit a range of phenotypes: (A) no visible defects, (B) gross defects of the body axis and general disruption of development, (C) truncated, thickened, or crooked intersomitic vessels (arrows), abnormal protrusion from body axis (arrowhead), (D) open neural tube with accompanying disruption of vascular plexus of the head. (E-G) E9.5 RasGRP3$^{gt/gt}$ embryos dissected from mothers with blood glucose levels in excess of 600 mg/dL (33mM) (E,G) and of 552 mg/dL (31mM) (F). RasGRP3$^{gt/gt}$ embryos generally either appear normal (E,F), or have vascular defects only in the head plexus (F). (H-J) PECAM-stained yolk sacs from (H) normoglycemic, (I) diabetic wildtype, or (J) diabetic RasGRP3$^{gt/gt}$ mice.

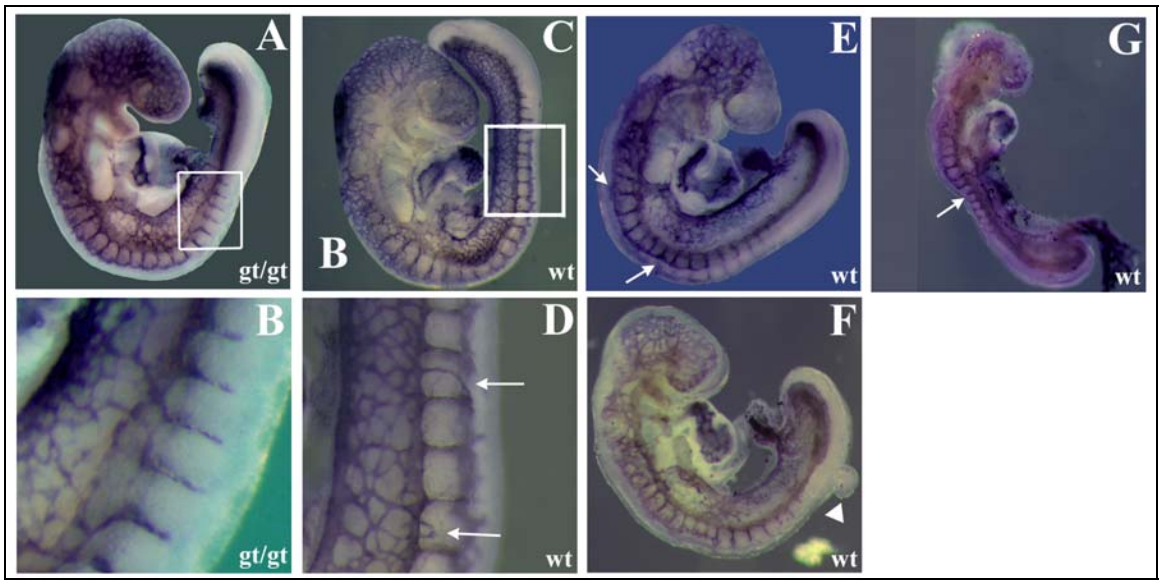

Figure 5: RasGRP3$^{gt/gt}$ embryos are less susceptible than wildtype embryos to defects induced by maternal diabetes on the C57BL/6J inbred genetic background. A-G: E9.5 PECAM antibody-stained embryos, (A) RasGRP3$^{gt/gt}$ embryo dissected from mother with blood glucose level of 376 mg/dL (21 mM), (B) magnified view of outlined portion of 6A. (C) Wildtype embryo dissected from mother with blood glucose level of 482 mg/dL (27mM) and exhibiting intersomitic vessel defects, (D) magnified view of outlined portion of 6C: white arrows indicate aberrant vessel growth in somites. (E) Wildtype embryo dissected from mother with blood glucose level of 392 mg/dL (22 mM) and exhibiting neural tube closure and intersomitic vessel defects (white arrows). (F,G) Wildtype embryos dissected from mother with blood glucose level of 513 mg/dL (29 mM) and exhibiting (F) intersomitic vessel defects (white arrows) and growth of abnormal structure from dorsal aspect of tail (white arrowhead) and (G) neural tube closure defect and crooked body axis with intersomitic vessel defects (white arrow).

Genotype	Treatment	Head plexus defects alone	Head plexus defects + head deformed	Inter-somitic vessel defects	Non-vascular deformities	Average number of somites
$PECAM^{lacZ/+}$	Cultured w/ 20mM glucose	36% (4/11)	27% (3/11)	27% (3/11)	27% (3/11)	19.2
$PECAM^{lacZ/+}$	STZ-induced diabetic mother	25% (9/36)	33% (12/36)	81% (29/36)	44% (16/36)	21.3
$RasGRP3^{gt/gt}$	Cultured w/ 20mM glucose	0% (0/17)	0% (0/17)	no data*	0% (0/17)	18.8
$RasGRP3^{gt/gt}$	STZ-induced diabetic mother	8% (1/12)	0% (0/12)	0% (0/12)	8% (1/12)	26.6

Table 5: Comparison of defects in E9.5 wildtype and $RasGRP3^{gt/gt}$ embryos from STZ-induced diabetic mothers or cultured with 20mM glucose for 24 hours.

*: Intersomitic vessels could not be analyzed in these embryos due to somitic expression of RasGRP3 driving β–gal expression.

Genotype	Blood glucose levels	Head plexus defects alone	Head plexus defects + head deformed	Inter-somitic vessel defects	Non-vascular deformities	Average somite number
wildtype	normoglycemic (avg. 182 mg/dL)	0% (0/9)	0% (0/9)	0% (0/9)	0% (0/9)	39.3
wildtype	diabetic (avg. 416 mg/dL)	3% (1/30)	20% (6/30)	71% (22/31)	45% (14/31)	23.3
RasGRP3$^{gt/gt}$	normoglycemic (avg. 216 mg/dL)	0% (0/9)	11% (1/9)	11% (1/9)	11% (1/9)	33.4
RasGRP3$^{gt/gt}$	diabetic (avg. 319 mg/dL)	0% (0/18)	0% (0/18)	17% (3/18)	6% (1/18)	21.9

Table 6: RasGRP3$^{gt/gt}$ embryos are less susceptible than wildtype embryos to defects induced by maternal diabetes on the C57BL/6J inbred genetic background.

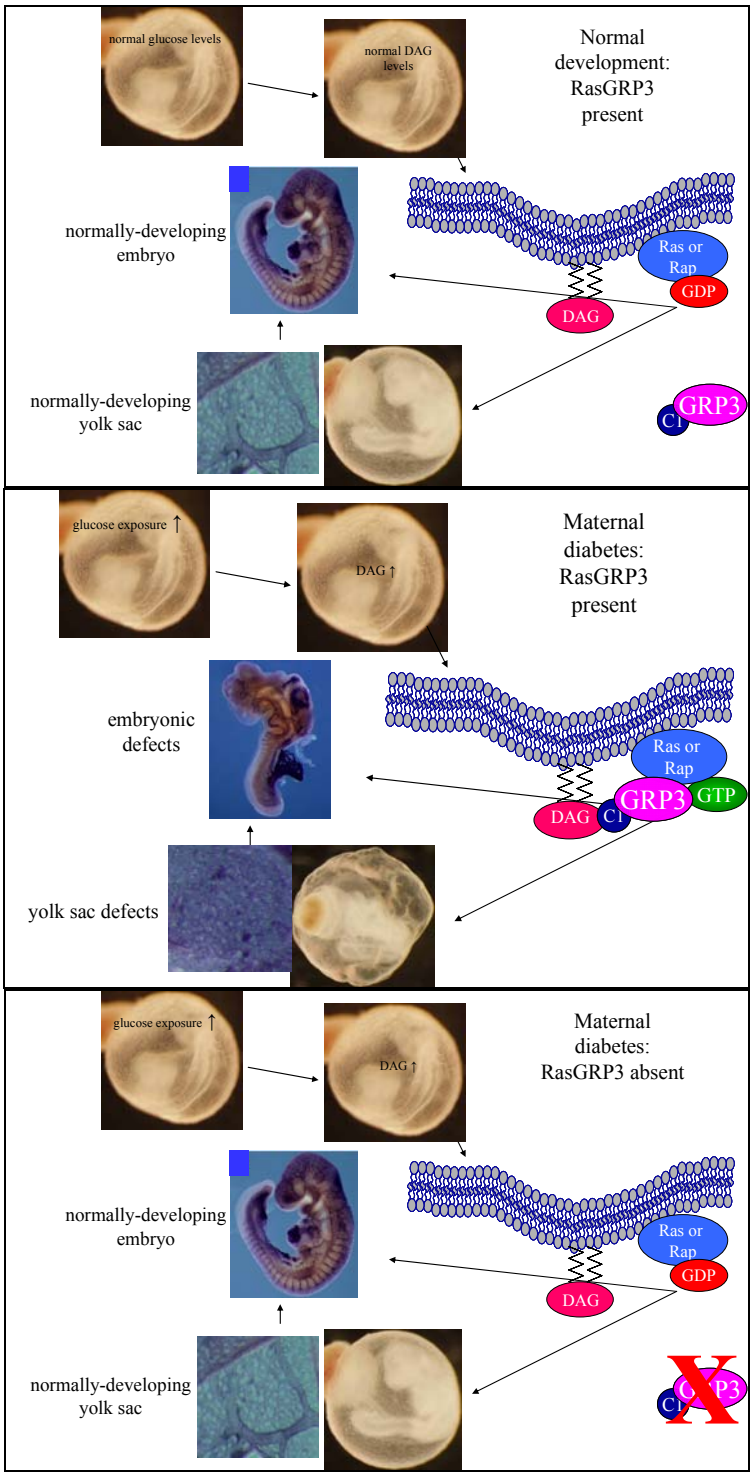

Figure 6: Model of impact of disrupted glucose or DAG signaling events on wildtype or RasGRP3$^{gt/gt}$ embryos compared to normal development.

CHAPTER 4: DISCUSSION

4.1. Effects of RasGRP3 on the embryonic response to phorbol ester

My research has provided strong evidence that RasGRP3 is required for the embryonic response to phorbol esters. My initial experiments using wildtype and RasGRP3$^{gt/gt}$ embryos from different litters suggested this scenario, and experiments culturing embryos from the same RasGRP3 intercross litter with PMA confirmed this result. I believe that the many defects exhibited by wildtype embryos treated with PMA are in large part secondary to those induced by PMA in their extraembryonic membranes, including vascular defects in the yolk sac. Blood vessels begin developing in these yolk sacs, but regulation of this formation appears to be disrupted to allow formation of dramatically enlarged and irregular vessels that do not establish a functional circulation. This phenotype has also been observed in yolk sacs of mice with a genetic deletion of vascular endothelial cell-specific tyrosine phosphatase (VE-PTP), a protein that has been found to be required for maintenance and remodeling of the primitive vascular plexus to establish functional and properly arborized yolk sac vasculature (Baumer et al. 2006). PMA-treated wildtype yolk sacs were able to successfully establish at least a primitive vasculature, so it is likely that the vascular defects in these yolk sacs are a result of misregulation of some remodeling step.

A mechanism by which these enlarged vessels could form is through failure of the process of intussusceptive angiogenesis. This is the process by which a large vessel grows a new wall through its lumen to split into two smaller vessels, and it has been

shown in multiple locations (including the yolk sac) to be an important complement to sprouting/branching angiogenesis (Djonov et al. 2003). Vessel fusion could also contribute to these abnormally large vessels. If VEGF signaling is not properly regulated, hyperfusion of vessels can occur to result in exceedingly large vessels (Drake and Little 1995). A third possibility is that the cells of the yolk sac vasculature are dividing more rapidly when exposed to PMA, resulting in larger vessels. To determine which, if any, of these mechanisms contributes to the formation of the enlarged vessels in PMA-treated wildtype yolk sacs, closer analysis of these yolk sacs will be required. Cell divisions in the yolk sac could be monitored using BrdU labeling. To determine whether vessel hyperfusion or failure of intussusception occurs in these yolk sacs would require measuring some of the more easily-recognizable vessels at intervals throughout the 24-hour PMA treatment period. If, over the 24 hours, a vessel continuously grows larger in both control and PMA-treated yolk sacs, then the grossly-enlarged PMA-induced vessels are likely due to vessel hyperfusion. If timepoints are frequent enough, fusion between two neighbouring vessels could potentially be observed. However, if a vessel measured in control yolk sacs grows to a certain point and then decreases in size as a result of intussusceptive growth or some other remodeling process while the same vessel in a PMA-treated yolk sac continues to simply grow bigger, then the enlarged vessel is due to a failure of intussusception or remodeling.

The larger size of these vessels could affect embryonic development through changes in blood flow dynamics, as the velocity of blood flow might be reduced in the yolk sac due to the lack of small vessels. In addition to reduced flow, these large vessels could have altered vascular permeability. Increased angiogenic growth of vessels is

accompanied by an increase in vascular permeability in many pathological conditions (for review: Byrne et al. 2005). Misregulation of VEGF signaling can lead to increased vascular permeability via signaling through several pathways, one of which requires DAG activation (for review: Bates and Harper 2002). Changes in permeability could impact the nutrients reaching the embryo, resulting in embryonic defects. Alterations in yolk sac vascular permeability could potentially be assessed by injection of colored dye into the yolk sac vessels or heart to determine whether this is a factor in wildtype or RasGRP3$^{gt/gt}$ embryos cultured with PMA.

The mouse embryo is not completely dependent on yolk sac circulation for survival at the stages I analyzed. This explains why the yolk sac defects I observed were not lethal. However, I believe that while not yet lethal, the severity of these partially RasGRP3-dependent primary defects in the yolk sac is enough to disrupt the embryo's normal environment, causing secondary defects in the embryo proper. RasGRP3$^{gt/gt}$ embryos, with their less severe yolk sac vascular defects, are unaffected by PMA treatment, suggesting that the vessels of the yolk sac may need to be disrupted and enlarged past some critical point in order to cause defects in the development of the embryo. Thus, although the vessels of RasGRP3$^{gt/gt}$ yolk sacs treated with PMA are larger and more irregular than those of controls, they may not be so irregular as to actually disrupt development of the embryo, allowing RasGRP3$^{gt/gt}$ embryos to appear normally-developed even when treated with 100nM PMA. It would be interesting to measure the average vessel diameter in yolk sacs treated with PMA to determine if there is a cutoff point of yolk sac vessel size that leads to defects in the embryo proper. With their range of phenotypes when exposed to PMA, RasGRP3$^{gt/+}$ embryos would be the

ideal genotype to analyze for this purpose. If the most normal embryos have the smallest yolk sac vessels while the most abnormal embryos have the largest vessels, it would definitely suggest that the abnormally large vessels of the yolk sac are at least partially responsible for the defects in the embryo proper.

Further experiments are required to more fully characterize the yolk sac defects caused by PMA. These experiments include hematoxylin and eosin (H&E) staining and PECAM antibody-staining of these yolk sacs. H&E staining will show the yolk sac defects on a cellular level, as hematoxylin stains nuclei and eosin stains the cytoplasm. PECAM antibody-staining will help elucidate the vascular-specific impact of PMA treatment. These two staining methods will help us to interpret the PMA-induced gross morphological defects we see in the yolk sac and to further understand how they might contribute to the failure to establish a functional yolk sac circulation.

One further point of interest in the response of RasGRP3 intercross embryos to PMA is the intermediate phenotype exhibited by RasGRP3$^{gt/+}$ embryos. We had not previously looked at the response of heterozygous embryos to PMA, so this intermediate phenotype was particularly interesting. RasGRP3$^{gt/+}$ ES-derived vessels show an intermediate response to PMA compared to RasGRP3$^{gt/gt}$ or wildtype ES-derived vessels (Roberts et al. 2004), and my work showed that this is also the case in whole embryos. This provides further evidence that RasGRP3 functions in a dosage-dependent fashion to mediate the response to PMA treatment.

4.2. Effects of RasGRP3 on the embryonic response to elevated glucose levels *in vitro*

Although investigations of the role of RasGRP3 on the response to embryo culture with glucose were not extensive, my results suggest that RasGRP3 is required to mediate this response as well. I do not have as much insight into the origin of glucose-induced defects as I had into the origin of PMA-induced defects. This was primarily because of the fact that these experiments were concluded before the importance of the yolk sac became apparent in the experiments with PMA. Therefore, in future experiments, it will be important to examine the effects of glucose treatment on the yolk sac.

In wildtype embryos cultured with 20mM glucose, the vessels of the yolk sac are enlarged and irregular (Pinter et al. 1999). This phenotype is similar to but less severe than what I saw in PMA-treated wildtype yolk sacs. If yolk sac defects are, in fact, contributing to embryonic defects, then this reduced severity of yolk sac vascular defects in glucose-treated compared to PMA-treated embryos could explain the more dramatic defects seen in PMA-treated embryos. This prediction is supported by the fact that wildtype embryos treated with glucose for 24 hours are still alive and still have a functional yolk sac circulation at E9.5. This would be impossible for wildtype PMA-treated embryos at this stage of development because even at E8.5 their yolk sacs are so severely impacted by the PMA that they do not have a functional circulation and so would most likely be dead before E9.5.

Based on previous work that showed elevated glucose levels and increased glucose metabolism increased *de novo* synthesis of DAG (Craven 1990), I hypothesized that treating embryos with glucose induced upregulation of DAG levels in these embryos.

Because PMA functions as a DAG analog, the functional significance is that treatment with glucose is predicted to manipulate the pathway further upstream of RasGRP3 than would treatment with PMA, which is directly upstream of RasGRP3 activation. Manipulating this pathway at a point further upstream would give any resultant effects more relevance and applicability to a true *in vivo* situation, but it could also lead to either more non-specific effects or, as I saw, more subtle defects than direct manipulation via PMA treatment. This could be due to activation of pathways and molecules in addition to RasGRP3 and/or to compensatory or checkpoint mechanism activation to reduce the severity of the response.

These experiments were performed comparably to the initial PMA experiments. Therefore, to be entirely confident in these results, it would be necessary to perform more stringently-controlled experiments, similar to the RasGRP3 intercross experiments I performed to confirm the PMA results. However, I sought to determine the role of maternal diabetes in development *in vivo*, using the streptozotocin-induced diabetic model of type I diabetes, discussed below.

4.3. Effects of RasGRP3 on the embryonic response to streptozotocin-induced diabetes

RasGRP3$^{gt/gt}$ embryos experience less severe vascular and nonvascular defects than do wildtype embryos when growing in a diabetic maternal environment. PECAM antibody-staining of yolk sacs was somewhat inconsistent due to procedural problems. However, I achieved sufficient staining of the yolk sac vessels in these experiments to be able to state with confidence that the vessel patterning of the yolk sacs of some wildtype

embryos from diabetic mothers is abnormal compared to those from normoglycemic mothers or compared to any RasGRP3$^{gt/gt}$ yolk sacs. These abnormal yolk sacs show disruption of the normal arborized pattern of the vasculature.

In addition to the VE-PTP mutant mice discussed earlier (see chapter 4.1), there are numerous genetic knockout mice that have severe defects in the yolk sac and other extraembryonic membranes, and many of these knockouts are genes important in cell adhesion. I believe this is one area that could prove essential to understanding how yolk sac dysmorphogenesis occurs in embryos treated with PMA or grown in a diabetic environment, and investigating a role for RasGRP3 in mediating cell-cell contacts is an ongoing project in our lab. Fibronectin is important in early vascular events in the yolk sac, and its loss results in failure of the yolk sac mesoderm and endoderm to fuse, leading to complete failure of blood vessel plexus formation in the yolk sac (George et al. 1993). Vascular cell adhesion molecule 1 (VCAM-1) is important early in extraembryonic tissue development, where it is essential for proper patterning of these tissues, as well as later for development of functional umbilical vessels (Kwee et al. 1995). Other cell adhesion molecules are also critical in these processes, including α4-integrins that can interact with VCAM-1 for proper development of the extraembryonic membranes (Yang et al. 1995). In human endothelial cells, phosphorylated PECAM interacts with β1-integrins to tighten cell-cell contacts and reduce migration and proliferation (Lu et al. 1996). These and other results provide a solid body of work highlighting the importance of regulation of cell-cell contacts for proper vascular development in the yolk sac and elsewhere.

RasGRP3 has been shown *in vitro* to activate both Ras and Rap1 (Rebhun et al. 2000), both of which have downstream targets controlling junctional stability. Increasing

RasGRP3 activation by exposing cells to elevated PMA (a RasGRP3 activator) or to elevated glucose which increases levels of DAG (another RasGRP3 activator) could increase downstream activation of Ras and/or Rap1 to modulate junctional stability. Because destabilization of junctions is associated with migration and proliferation as well as with disease conditions such as tumor angiogenesis, I would predict that in the diabetic condition junctions are destabilized as well. Because increased Ras activation leads to decreased junctional stability, the level of Ras activation could be examined by Western blot analysis of homogenized yolk sac tissues. If the yolk sac vascular phenotype is characterized by a decrease in junctional stability, I would expect to see an increase in Ras activation, whereas increased junctional stability would be marked by an increase in Rap1 activation.

Additionally, there is evidence in the ES cell model that the major vascular adherens junction component VE-cadherin is mislocalized to the cytoplasm during PMA-induced vascular dysmorphogenesis and that this mislocalization is RasGRP3-dependent (P. Randhawa, unpublished results). VE-PTP has been shown to act on VE-cadherin to increase its stability in junctions and reduce vascular permeability (Nawroth et al. 2002). This is interesting, because, as previously mentioned, the vascular phenotypes of VE-PTP knockout embryos bear many striking similarities to those I see in wildtype embryos treated with PMA or grown in a diabetic mother. Analysis of VE-cadherin localization during PMA- or diabetes-induced embryonic vascular dysmorphogenesis could determine whether alterations in the adherens junctions are important in this system as well.

Defects induced in the yolk sacs of these embryos may disrupt the embryo's environment enough to cause defects such as failure of neural tube closure and

mispatterning of small vessels such as intersomitic vessels and head plexus vessels. In addition to abnormally large yolk sac vessels, VE-PTP mutant mouse embryos have defects in the head plexus and intersomitic vessel formation that are most likely attributable to the defects in the yolk sac (Baumer et al. 2006). These angiogenic events, as well as nonvascular events such as neural tube closure, are all processes that occur during the stages of development assayed, making them susceptible to defects induced by improper establishment of yolk sac circulation during the same time period. In fact, many of these defects have been found to be very temporally specific and will only occur if hyperglycemic conditions are present during a narrow window of time between E8.0 and E9.5 (Pinter et al. 1999).

When RasGRP3$^{gt/+}$ female mice were made diabetic, the embryos showed very few defects. While this was disappointing in terms of not adding support to my results on outbred mice, it is very interesting because it suggests that the genotype of the mother may play a role in the response of the embryo to maternal diabetes. RasGRP3 is re-expressed in maternal blood vessels undergoing neoangiogenesis during pregnancy. Therefore, it may be the case that RasGRP3$^{gt/gt}$ mothers confer protection to their RasGRP3$^{gt/gt}$ offspring, while wildtype mothers (who express RasGRP3 in the developing vessels that support the embryos) contribute to or exacerbate embryonic defects. RasGRP3$^{gt/+}$ mothers, with a lower level of RasGRP3 expression in these maternal vessels, may also confer protection to the developing embryos, regardless of their genotype. It would be interesting to investigate this result in more detail. One possible approach to this question could be to implant wildtype embryos in a diabetic RasGRP3$^{gt/gt}$ mother, or vice versa. If wildtype embryos developing in a RasGRP3$^{gt/gt}$

mother have less severe defects than wildtype embryos developing in a wildtype mother, it would support the idea that the mother's genotype is a key determinant of an embryo's response to maternal diabetes. Maternal tissues could also be examined for diabetes-induced vascular defects, either in the decidua at the stages investigated or later in development, once the placenta is established. Differences between diabetic wildtype and diabetic RasGRP3$^{gt/gt}$ maternal vessels could also provide insight into how RasGRP3 affects embryonic vascular development.

In the inbred B6 mice, defects in moderately-diabetic wildtype embryos were less severe than those seen in the extremely diabetic outbred mice. This can be explained by previous work that showed vascular defects directly correlate with the level of hyperglycemia (Algenstaedt et al. 2003). Further support for this is observed in the severe defects seen in a wildtype B6 litter that had a blood glucose level comparable to that of the outbred mice. It will take more time to compile greater numbers to confirm the results observed in this litter because of the reproductive problems documented in B6 mice and further compounded in diabetic mice (Levine 1965; Pinter et al. 1999). While none of the RasGRP3$^{gt/gt}$ B6 mice had blood glucose levels exceeding 500 mg/dL, there were no defects in any embryos from moderately diabetic (blood glucose levels of 300-500 mg/dL) mothers. Thus, while it would be best to have embryos from an extremely diabetic mother to support the finding that RasGRP3$^{gt/gt}$ embryos are refractory to diabetes-induced defects, I believe that my results up to this point offer fairly convincing evidence that this is the case.

In conclusion, it is clear from my results that RasGRP3 is critical in mediating disrupted signaling events during embryogenesis. During normal embryogenesis,

RasGRP3 is not required for proper development, but when DAG levels are elevated in a pathological condition such as diabetes, increased activation of RasGRP3 may contribute to vascular dysmorphogenesis. Vascular and nonvascular defects experienced in these disease states are at least in part secondary to defects in the yolk sac vasculature (Figure 6). Future work will expand on this model and explore the mechanisms and specific role of RasGRP3 in mediating these yolk sac vascular defects and their impact on the embryo, including potentially increased vascular permeability or decreased cell-cell adhesion.

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