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SOLUBLE GUANYLYL CYCLASE ACTIVATION PROMOTES ANGIOGENESIS

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Running Title: sGC in angiogenesis

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Abbreviations: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ); 5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine (BAY 41-2272); chicken chorioallantoic membrane (CAM); endothelial cells (EC); extracellular signal regulated kinase (ERK); green fluorescent protein (GFP); hours post fertilization (hpf); human umbilical vein endothelial cells (HUVEC); mitogen activated protein kinase (MAPK); nitric oxide (NO); vascular endothelial growth factor (VEGF); sodium nitroprusside (SNP); soluble guanylyl cyclase (sGC)

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Abstract

Soluble guanylyl cyclase (sGC) is a cGMP-generating enzyme, carrying a heme prosthetic group that functions as a nitric oxide (NO) sensor. sGC is present in most cells types, including the vascular endothelium, where its biological functions remain largely unexplored. Herein, we have investigated the role of sGC in angiogenesis and angiogenesis-related properties of endothelial cells (EC). Initially, we determined that sGC was present and enzymatically active in the chicken chorioallantoic membrane (CAM) during the days of maximal angiogenesis. In the CAM, inhibition of endogenous sGC inhibited neovascularization, while activation promoted neovessel formation. Using zebrafish as a model for vascular development, we did not detect any effect on vasculogenesis upon sGC blockade, but we did observe an abnormal angiogenic response involving the cranial and intersegmental vessels, as well as the posterior cardinal vein. In vitro, pharmacological activation of sGC, or adenovirus-mediated sGC gene transfer promoted EC proliferation and migration, while sGC inhibition blocked tube-like network formation. In addition, sGC inhibition blocked the migratory response to vascular EC growth factor. Cells infected with sGC-expressing adenoviruses exhibited increased ERK1/2 and p38 MAPK activation that was sensitive to sGC inhibition by ODQ, suggesting that these MAPKs are downstream effectors of sGC in EC. A functional role for p38 in cGMP-stimulated migration was demonstrated using SB203580; pharmacological inhibition of p38 attenuated BAY 41-2272- and sGC overexpression-induced EC mobilization. We conclude that sGC activation promotes the expression of angiogenesis-related properties by EC and that sGC might represent a novel target to modulate neo-vessel formation.

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Introduction

Angiogenesis, the formation of new blood vessels from pre-existing structures, is a highly orchestrated process that requires degradation of the extracellular matrix, proliferation and migration of endothelial cells, followed by organization of the EC into stable patent structures supported by mural cells(Folkman and Shing, 1992;Conway et al., 2001). In the adult, angiogenesis is tightly regulated with vessel growth being limited to a few tissues; deregulated angiogenesis has been proposed to contribute to several disease processes including tumor growth, psoriasis, arthritis and diabetic retinopathy(Carmeliet, 2003). During angiogenesis, EC integrate signals form various soluble and matrix-bound molecules to form new vessels(Bischoff, 1997;Conway et al., 2001). Among the endogenous mediators proposed to play an important role in neovascularization, is the labile diatom molecule nitric oxide (NO)(Morbidelli et al., 2003). Exogenously applied NO donors have been shown to stimulate EC growth and migration in vitro(Ziche et al., 1994;Isenberg et al., 2005); moreover, endogenous NO mediates many of the effects of the prototype angiogenic factor vascular endothelial growth factor (VEGF)(Papapetropoulos et al., 1997;Ziche et al., 1997). In vivo, ischemia-induced angiogenesis was attenuated in eNOS knockout animals and VEGF-stimulated vessel formation in the cornea could be blocked by administration of the NOS inhibitor L-NAME(Ziche et al., 1997;Murohara et al., 1998). However, in spite of the wealth of information of the angiogenic effects of NO, the downstream pathways mediating its effects remain poorly characterized.

Soluble or NO-sensitive guanylyl cyclase is a ubiquitously expressed enzyme that acts as a “receptor” for NO(Hobbs, 1997). The low basal activity of sGC increases several hundred-fold upon activation with NO converting GTP to cGMP(Lucas et al., 2000).

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The most common form of the obligate sGC heterodimer is α1/β1 that is present in large amounts in smooth muscle, nerve cells and platelets(Hobbs, 1997). In the vascular system, the biological role of sGC has been mostly studied in the context of smooth muscle tone and platelet aggregation(Lucas et al., 2000;Friebe and Koesling, 2003). We have previously shown that sGC subunit mRNA and catalytic activity are also present in endothelial cells from different vascular beds(Papapetropoulos et al., 1996); however, the physiological role of sGC in vascular endothelium remains for the most part unexplored. With respect to angiogenesis, some evidence for the involvement of sGC exists as NO-stimulated EC proliferation is cGMP-dependent and a cell-permeable analogue of cGMP promotes EC migration(Parenti et al., 1998;Kawasaki et al., 2003). The aim of the present study was to characterize the contribution of sGC in neovessel formation; to this end, we studied the effects of pharmacological activators and inhibitors of sGC in two in vivo models; moreover, we sought to determine whether alterations in sGC levels or activity affect the migratory and proliferative potential of EC and to elucidate the pathway(s) involved.

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Methods

Materials

Cell culture media and serum were obtained from Life Technologies GIBCO-BRL (Paisley, UK). All cell culture plasticware was purchased from Corning-Costar Inc. (Corning, NY); cGMP enzyme immunoassay kits were from R&D Systems (Minneapolis, MN); SuperSignal West Pico chemiluminescent substrate from Pierce Biotechnology (Rockford, Illinois); *DC* Protein assay kit, Tween 20 and other immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA); jetPEI transfection reagent was obtained from Polyplus-transfection (Illkirch, France); penicillin and streptomycin from Applichem (Darmstadt, Germany); amphotericin, gentamycin and heparin were purchased from Biochrom AG (Berlin, Germany); the α_1 and β_1 sGC subunit antibodies, isobutylmethylxanthine (IBMX), N^G-Nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), bovine serum albumin, EDTA, ODQ, zaprinast and all protease inhibitors were purchased from Sigma-Aldrich (St Luis, MO); BAY 41-2272 was obtained from Qbiogene-Alexis GmbH (Grunberg, Germany); ERK1/2 and p38 phospho-specific and total antibodies along with the secondary antibodies were obtained from Cell Signalling Technology (Beverly, MA); luciferase reporter assay system was purchased from Boehringer-Mannheim (Mannheim, Germany); Leghorn fertilized eggs from Pindos (Iperos, Greece).

CAM angiogenesis assay

Fertilised White Leghorn chicken eggs were placed in an incubator and kept under constant humidity at 37^{o}C. On day 4, a square window was opened in the shell and then sealed with adhesive tape. On day 9, an O-ring (1 cm^2) was placed on the surface

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of the CAM and the tested substances (ODQ, BAY 41-2272 or zaprinast) were placed inside this restricted area. After 48 hr, CAMs were fixed in Carson's solution (saline-buffered formalin) and angiogenesis was evaluated using image analysis software as follows. CAM tissues were photographed using a digital camera attached to a steroscope and then imported into the Scion Image software. Photomicrographs were then transformed to binary files, thresholded and skeletonised. Vessel length was measured in pixels and expressed as percent of control.

Zebrafish lines and pharmacological treatment

Zebrafish were raised under standard laboratory conditions at 28°C. We used embryos from an incross of double heterozygous carriers for the following transgenes: the panendothelian gfp line *Tg(flk1:EGFP)*^{s843} (Jin et al., 2005) and the erythrocyte specific DsRed line *Tg(gata1:DsRed)(*Traver et al., 2003*)*. Embryos were incubated with 5 or 10 µM ODQ in embryo water. Analyses shown here were carried out with 5µM ODQ. Control embryos were incubated in 0,05% DMSO in embryo water. Embryos were fixed in 4%PFA overnight and then imaged.

Luciferase activity

African green monkey COSm6 (2 x 10^5 cells) were plated in 6-well plates and grown overnight. Cells were then transfected using the jetPEI transfection reagent (3 µg DNA and 6 µl of jetPEI per well). After 24 h, cells were collected and approximately 1x10^4 cells added inside each O-ring at 6, 9 and 12 days of embryogenesis. Following a 48hr incubation, CAM tissues were removed and assayed for activity using the luciferase reporter assay system according to the manufacturer's instructions.

Western blotting

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Proteins from tissues or cells were extracted after homogenization in a lysis buffer containing 1% Triton-X, 1% SDS, 150mM NaCl, 50mM NaF, 1mM Na_3VO_4, 0.5% sodium deoxycholate, 1mM EDTA, 0,1mM EGTA and protease inhibitors (10 μg/ml aprotinin, 10 μg/ml pepstatin, and 20 mM PMSF). Samples were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and incubated with the primary and secondary antibodies. Immunoreactive proteins were detected using a chemiluminescent substrate. Bands on autoradiographs were quantified using the Scion Image Release Beta 4.0.2 software. In case of double bands, as for example with ERK1/2, intensity from the two individual bands was calculated as one.

cGMP Enzyme Immunoassay

CAM tissues were removed and washed in Hank's balanced salt solution (HBSS). They were then incubated with the NOS inhibitor L-NAME for 10min; tissues were then stimulated with sodium nitroprusside (100 μM) in the presence of the phosphodiesterase inhibitor IBMX (1 mM) for 20 min. Media were then aspirated and tissues were homogenised in 0.1 N HCl to extract cGMP. After 30min HCl extracts were collected and cGMP was analysed using a commercially available enzyme immunoassay kit following the manufacturer's instructions.

Construction of adenoviral plasmids and production of adenoviruses. The adenoviral plasmids used in our study were constructed using standard methodology. Briefly, rat α1 and β1 cDNAs were subcloned into the pShuttle-CMV vector and recombined with pAdeasy-1 in BJ5183 cells. Recombinants were identified via restriction analysis and transfected into HEK cells (2×10^6) using the jetPEI reagent. Replication incompetent adenoviruses were then propagated in HEK cells and titered using the

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cytopathic effect assay. HUVEC were infected with Ad-sGCα1 (20 moi) and Ad-sGCβ1 (20 moi each) or Ad-GFP (40 moi) as control. After 20-48 h of infection, the infected cells were used for biochemical analyses, or trypsinised and used in migration, proliferation and tube-formation assays.

HUVEC cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh cords and grown on 100-mm dishes in M199 supplemented with 15 % bovine calf serum, 50 U/mL penicillin, 50 µg/mL streptomycin, 50 µg/ml gentamycin, 2.5 µg/ml amphotericin B, 5 U/ml sodium heparin and 150-200 µg/ml endothelial cell growth supplement. HUVEC between passages 1 and 2 were used for all experiments.

Matrigel® in vitro tube-formation assay

The formation of tube-like structures by HUVEC cells was assessed on growth factor–reduced Matrigel®. HUVEC were plated at 15,000 cells/well in 96-well plates, pre-coated with 45 µL of Matrigel® in the presence of ODQ (10µM), BAY 41-2272 (1µM) or DMSO as vehicle. After 24 hr of incubation, tube formation was quantified. In brief, phase-contrast photomicrographs of endothelial cell cultures were recorded and imported into the freeware image analysis program Scion Image (Release Beta 4.0.2, Scion Corporation). Images were converted to a binary format and the binary threshold was adjusted to obtain the best contrast of tubules. The images were then skeletonised and total tubule length was measured in pixels. The area occupied by aggregates of cells was considered as noise. The total length calculated was then expressed as a precent of control.

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Cell proliferation

HUVEC cells were seeded in 24-well plates at $6x10^3$ cells/cm^2 and incubated in M199 supplemented with FBS and ECGS for 24 hours. Cells were incubated with the indicated concentration of BAY 41-2272 (0.1 and 1 μM) and allowed to proliferate for 48 hr. After this time they were trypsinised and cell number determined using a hemocytometer.

Cell migration

Cells were serum-starved overnight. After trypsinization $1x10^5$ cells were added to transwells (8 μM pore size) in 600 μl of serum-free medium containing 0.25% BSA. The test compound (8Br-cGMP 1mM; BAY 41-2272 1μM; VEGF 50ng/ml plus or minus ODQ 10μM) or vehicle was added to the well containing the transwell inserts. To inhibit p38, cells were pre-incubated with SB203580 (10μM) for 30min. HUVEC were allowed to migrate for 4 hr; after this time non-migrated cells at the top of the transwell filter were removed with a cotton swab. The migrated cells were fixed in Carson's solution for at least 30 min at room temperature and then stained in toluidine blue for 20 minutes at room temperature. Migrated cells were scored in 8 random fields.

Data Analysis

Data are expressed as means $\pm$ SEM of the indicated number of observations. Statistical comparisons between groups were performed using ANOVA followed by a post-hoc test, or Student's t-test, as appropriate. Differences were considered significant when $p<0.05$.

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Results

Pharmacological manipulation of sGC activity results in altered angiogenic responses in vivo. To determine if sGC subunits are expressed in tissues exhibiting an active angiogenic response, we performed western blot analysis of extracts from CAM tissue. In these experiments, both subunits of the most common sGC isoform (α1/β1) were detected (Fig.1A); α1 and β1 levels were developmentally regulated, showing peak expression during days 9-12.

Using a heterologous system we demonstrated that endogenous sGC protein levels correlate with α1 promoter activity (Fig.1B). COSm6 cells were transfected with an empty luciferase vector (pGL3) or a vector in which luciferase was expressed under the control of the sGC α1 promoter (Vazquez_Padron et al., 2004); cells were then placed on the CAM. Growth factors and other mediators produced in CAM tissue diffuse and modulate α1 promoter activity in the COSm6 cells. In these experiments we observed a time-dependent increase in luciferase activity that mirrored the protein expression of α1. Some of the regulatory sequences in the α1 promoter required for expression reside within the 5' untranslatted region (5′ UTR), since deletion of this region resulted in reduced luciferase activity at all of the time points tested. To determine whether the sGC present in the CAM is catalytically active, we measured cGMP accumulation in the presence and absence of the NO donor sodium nitroprusside (SNP). Indeed, stimulation of CAM tissues with SNP led to a robust increase in cGMP levels (Fig.1C).

We next examined the impact of sGC inhibition in angiogenesis in the CAM. Incubation with the selective sGC inhibitor ODQ (Garthwaite et al., 1995)caused a

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dose-dependent inhibition of vascular length, suggesting that sGC is important in the formation of neovessels (Fig.1D). On the other hand, treatment of CAMs with the NO-independent sGC activator BAY 41-2272 or the phosphodiesterase 5 inhibitor zaprinast stimulated angiogenesis as indicated by the increase in CAM vessel length (Fig.1E). The increase in vessel length in the CAM in the presence of either BAY 41-2272 or zaprinast was approximately half of the response observed with 1μg of VEGF (30±9.1% increase over control).

In order to test if sGC participates in endothelial cell formation and vascular development in vivo we used zebrafish as a model organism (supplementary movie 1). Zebrafish embryos were treated with different concentrations of ODQ in embryo water and endothelial and erythrocyte birth and development were monitored in embryos that resulted from an incross of *Tg(gata1:DsRed); Tg(flk1:EGFP)*^{s843} double heterozygotes. When embryos were treated with 10μM ODQ concentration from 1 cell stage on, their development was arrested at gastrulation stage (data not shown). Inhibition of sGC using a lower ODQ concentration (5μM) added at 10hpf allowed normal formation and differentiation of EC; however, in these embryos we observed that pericardial edema developed, thinner intersegmental vessels existed and the posterior cardinal vein was disorganized (Fig. 2 B, D, E compare with A, C). Furthermore, there was a significant reduction in gata1:DsRed positive cells, which mark myeloid cells (Fig.2 D,E and supplementary movie 2). Finally, embryos treated with ODQ at later stages of development (24hpf) possessed fewer cranial vessels and their erythrocytes seemed to clot in the posterior part obstructing circulation (Fig.2 F); the above phenotypes were reversible upon removal of the sGC inhibitor. Our data,

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taken collectively, suggest that sGC is required for proper erythrocyte development and normal angiogenesis, at least in some vascular beds, in the zebrafish embryo.

sGC activation promotes the expression of an angiogenic phenotype in cultured endothelial cells. To determine whether sGC activation affects EC properties related to angiogenesis, we treated HUVEC with BAY 41-2272. Such treatment resulted in a concentration-dependent increase in EC proliferation (Fig.3A). In addition, incubation of HUVEC with BAY 41-2272, or the cell permeable analogue of cGMP 8Br-GMP, stimulated EC migration in vitro (Fig.3B). In a different series of experiments, we observed that HUVEC grown on Matrigel® in the presence of the sGC inhibitor ODQ formed fewer capillary-like structures (Fig.3C). To test if sGC is part of the signaling cascade activated by angiogenic factors, we employed vascular endothelial growth factor and measured EC migration in response to this growth factor, in the presence and absence of ODQ. VEGF stimulated migration approximately 4-fold in an ODQ-sensitive manner, suggesting that sGC mediates some of the angiogenic actions of this prototype angiogenic growth factor (Fig.3D).

Overexpression of sGC promotes EC proliferation, migration and tube-like structure formation. We next examined EC properties associated with new blood vessel formation in cells transduced with adenoviruses to overexpress the sGC subunits. After infection, HUVEC expressed significantly higher α1 and β1 protein levels and sGC activity as compared to uninfected or GFP-infected cells (data not shown). sGC overexpressing cells exhibited higher proliferation rates than control cells (Fig.4A). Similar results were obtained in migration assays where α1/β1 overexpressing cells showed a 2-fold greater basal migration rate as compared to cells infected with a

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GFP-expressing adenovirus and exhibited an augmented migratory response to VEGF (Fig.4B). Increased sGC expression also correlated with an increase in the ability of EC to form tube-like networks on Matrigel®; the increase in network formation (40%) was of similar magnitude to that seen with naïve EC stimulated with BAY 41-2272 (Fig.4C).

Mechanisms of sGC-triggered angiogenic responses. We next sought to determine the pathways involved in sGC-regulated responses that are relevant to angiogenesis. As MAPK members have been implicated in EC proliferation and migration, we tested whether sGC-overexpressing cells exhibit increased levels of ERK1/2 and p38 activation. Indeed, cells infected with viruses containing the sGC transgenes displayed a significantly higher pERK1/2/total ERK1/2 ratio indicating that in these cells ERK1/2 is activated (Fig.5A&B). Similar results were obtained for p38 (Fig.5A&B). Moreover, incubation of HUVEC with BAY 41-2272 stimulated ERK1/2 and p38 phosphorylation in a time-dependent manner (data not shown). Inhibition of sGC by ODQ resulted in a reduction in the phosphorylation of both MAPK members tested (ERK1/2 and p38) in cells infected with the α1 and β1 sGC subunits (Fig.5C&D), endogenous sGC inhibition by ODQ reduced only ERK1/2 activation in GFP-infected cells. Finally, to prove the functional relevance of p38 activation to sGC-triggered migration, we studied EC migration in the absence and presence of a p38 inhibitor (SB203580). Pharmacological inhibition of p38 attenuated the migration of both uninfected cells stimulated with BAY 41-2272, as well as the migration brought about by increased sGC expression (Fig.5E).

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Discussion

Although present in substantial amounts in EC, our knowledge on the role of sGC in EC biology is limited. In the present study, we set out to determine the role of sGC in EC properties associated with angiogenesis and to evaluate the contribution of sGC in blood vessel growth. Initial experiments showed that α1 promoter activity, α1/β1 immunoreactivity and sGC activity in the CAM were expressed in a manner that coincided with maximal angiogenic activity in this tissue (days 9-12)(Maragoudakis et al., 1988). Inhibition of endogenous sGC activity by ODQ resulted in an inhibition of angiogenesis, while incubation with the sGC activator BAY 41-2272 increased neovascularization. In line with what was observed with the sGC activator BAY 41-2272, phosphodiesterase 5 inhibition by zaprinast led to an increase in vessel length in the CAM. It should be noted that conflicting results exist regarding the role of endogenous sGC activator NO in the CAM; older studies (Pipili-Synetos et al., 1994)suggested that NO synthase inhibition stimulates angiogenesis, while more recent observations (Polytarchou and Papadimitriou, 2004)favor a pro-angiogenic role for NO in this system, in line with the angiogenesis-stimulating properties of NO in all other systems studied so far. The initially reported anti-angiogenic actions of NO in the CAM might relate to the fact that in the older studies the material (coverslip) used to deliver the NO synthase inhibitor itself caused an inflammatory response, thereby making the use of cortisone acetate necessary and complicating data interpretation.

The molecular mechanisms that govern vascular development are highly conserved across species. Common growth and transcription factors participate in blood vessel formation in fish, avians and mammals. For example, VEGF, a growth factor that

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signals through NO/cGMP, is critical for angioblast formation and differentiation into arterial endothelium in zebrafish(Nasevicius and Ekker, 2000). Similarly, EC are formed during development from angioblasts/endothelial precursors in response to VEGF-induced flk-1 activation in mice(Shalaby et al., 1995;Carmeliet et al., 1996) . We took advantage of the fact that zebrafish embryos are free-living in the external environment, transparent during development and their vascular system develops rapidly to study the effects of sGC inhibition in vascular development. To visualize the appearance of EC in vivo we utilized transgenic animals that expressed GFP under the control of the zebrafish flk-1 promoter. The GFP expression pattern is specific to the vasculature and recapitulates the endogenous expression of flk-1(Liao et al., 1997). Incubation of embryos with ODQ starting at 10hpf did not have an overt effect on vasculogenesis, as GFP positive cells appeared normally and formed vessels. It should be noted that cGMP is detectable in zebrafish embryos at 8 hpf and its levels double after 20 hpf(Holmqvist et al., 2004). Interestingly, embryos that had their sGC inhibited 10-24 hpf displayed fewer cranial vessels, thinner intersegmental vessels and a disorganized posterior cardinal vein, indicative of an abnormal angiogenic response.

In order to evaluate the direct effects of sGC activation on EC properties that are important for neovascularization, we used HUVEC, exposed them to BAY 41-2272 and determined their proliferation rate. BAY 41-2272 is a new NO-independent heme-dependent activator of sGC(Stasch et al., 2001). We chose to use this agent to enhance sGC activity and increase intracellular cGMP levels over NO-generating cGMP elevating agents since we wanted to eliminate any cGMP-independent actions of NO donors, like S-nitrosylation, tyrosine nitration, interaction with heme- and non-

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heme iron containing proteins or interaction with lipids and free radicals that could contribute to or modify the cGMP-mediated responses(Davis et al., 2001). Exposure of endothelial cells to BAY 41-2272 increased EC number in a concentration-dependent manner. Our observations are in line with those of Isenberg et al., who demonstrated that NO-donors when used at low concentrations promote EC growth in a cGMP-dependent manner, while higher concentrations have cGMP-independent inhibitory effects on proliferation(Isenberg et al., 2005).

Another property of EC that is important for angiogenesis is the ability of these cells to organize into patent capillary structures. Using Matrigel® to drive network-like formation in vitro we observed that EC incubated with ODQ engaged less in network formation. In agreement to these results, we have previously shown that inhibition of endogenous NO production attenuates VEGF- and transforming growth factor β1-induced capillary-like structure formation in three dimensional collagen gels(Papapetropoulos et al., 1997). Finally, we tested whether activation of sGC or incubation with a cell-permeable analogue of cGMP affects the ability of EC to migrate. Both BAY 41-2272 and 8Br-cGMP promoted a 4-fold increase in EC migration in the absence of a growth factor, suggesting that sGC activation per se is sufficient to promote EC mobilization. Taken together, the data presented so far indicate that sGC plays an important role in all of the EC properties examined that are linked to angiogenesis. This conclusion is further strengthened by the observation that overexpression of sGC in EC using recombinant adenoviruses, increased the proliferation rate, migratory ability and organization of EC into network-like structures mimicking the responses obtained using pharmacological activators of the enzyme.

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VEGF is among the best characterized angiogenic factors(Ferrara et al., 2003;Zachary, 2003). Exposure of EC to VEGF increases NO production; the NO released then acts in an autocrine manner to promote angiogenesis(Papapetropoulos et al., 1997;Ziche et al., 1997;Ferrara et al., 2003). However, the contribution of cGMP to the angiogenic properties of VEGF hasn' t been fully explored. In one study, inhibition of sGC by ODQ blocked the increase in VEGF-stimulated EC proliferation(Parenti et al., 1998). To determine if cGMP formation also mediates the action of VEGF with respect to migration, we treated EC with ODQ and determined the migratory response to VEGF. ODQ-treated cells exhibited a blunted migratory response, suggesting that sGC activation is required for this classic growth factor to transmit some of its angiogenic signals. Moreover, our observation that cells infected with sGC adenoviruses exhibit an increased migratory response to VEGF lends further credence to the hypothesis that sGC plays an important role in mediating the biological responses to VEGF.

Mitogen activated protein kinase cascades have been linked to many biological responses associated with angiogenesis(Seger and Krebs, 1995). Members of this family of kinases become fully active after phosphorylation on both threonine and tyrosine residues(Johnson and Lapadat, 2002). EC proliferation in response to VEGF and other growth factors depends on ERK1/2 activation, while p38 has been shown to mediate the migration of EC(Zachary, 2003). Previous reports have shown that NO donors are capable of activating ERK1/2(Parenti et al., 1998;Oliveira et al., 2003). NO released from pharmacological concentrations of nitrovasodilators causes S-nitrosylation of Cys118 of p21Ras, leading to the recruitment and activation of

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several downstream kinases(Lander et al., 1997). On the other hand, cell-permeable analogues of cGMP have been proposed to activate Ras using a different, yet unidentified pathway(Oliveira et al., 2003). To investigate whether the EC proliferation observed in HUVEC overexpressing sGC correlates with an increase in ERK activation, we determined the phosphorylated/total ratio for this kinase. Indeed, higher sGC levels resulted in increased ERK1/2 phosphorylation in an ODQ-sensitive manner. ODQ also inhibited basal ERK1/2 phosphorylation, indicating that tonic production of cGMP contributes to basal activity of ERK1/2 in EC. Our data are in line with the observations that the NO-stimulated EC proliferation can be blocked by the MEK inhibitor PD98059(Parenti et al., 1998).

Although the role of ERK1/2 in proliferation is well established, the biological significance of p38 in EC has only recently started to be investigated(Parenti et al., 1998;Zachary, 2003;McMullen et al., 2005). Activation of the p38 MAPK by angiogenic growth factors has been shown to occur in vitro and proposed to play a role in their ability to stimulate migration(McMullen et al., 2004). In addition, overexpression of MEK6, an upstream activator of p38, promotes EC migration(McMullen et al., 2005). To find out whether activation of p38 occurs in cells overexpressing sGC, we used a phospho-specific Ab for this kinase to determime the phospharylation status of p38; indeed, higher ratios pp38/p38 ratios were observed in cells infected with sGC adenoviruses. The finding that the sGC inhibitor ODQ blocks the increase in p38 phosphorylation offers additional support to the hypothesis that increased cGMP levels promote p38 activation. To provide a functional link between p38 activation and sGC-driven migration, we studied the effect of SB203580 on the migratory behavior of cells exposed to BAY 41-2272 or

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cells infected with sGC expressing adenoviruses. In both cases, p38 inhibition attenuated the migratory response, suggesting that activation of p38 is at least in part responsible for this response.

In summary, we have shown that sGC contributes to several aspects of the angiogenic process by promoting EC proliferation, migration and organization in network-like structures. Moreover, we have demonstrated that sGC inhibition leads to abnormal/reduced angiogenic responses in two different systems in vivo. These actions of sGC in EC correlate with activation of at least two members of the MAPK signaling cascade known to regulate angiogenic responses, ERK1/2 and p38. We conclude that stimulation of sGC and the concomitant increase in cGMP exert a permissive role on blood vessel formation.

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Footnotes

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Legends for Figures

Figure 1. sGC activation promotes angiogenesis in the CAM. A. Western blot analyses for the α1 and β1 sGC subunits in lysates of CAM tissue isolated at different days of development. **B.** α1 promoter activity in the CAM. Luciferase activity was measured in the lysates of transfected COSm6 cells placed on the CAM for 2 days and harvested during different stages of development. Data are expressed as means ± S.E.M.; n=11-19; *p< 0.05 vs pGL3 (empty control vector); #p<0.05 vs Δ 5' UTR (untranslated region). 5' UTR: mouse α1 promoter containing the 5' UTR region; Δ 5'UTR: 5' upstream region without the UTR. **C.** sGC is catalytically active in the CAM. Tissues were removed at the indicated time after fertilization and incubated with L-NAME (100μM) to inhibit NOS-induced cGMP formation; after 10min they were stimulated with SNP (100μM). Data are expressed as means ± S.E.M.; n=8-16; *p< 0.05 vs L-NAME. **D.** sGC inhibition attenuates angiogenesis. CAMs were treated with the indicated dose of ODQ for 48hr; the total length of vessel network was determined using image analysis software. A representative photomicrograph showing reduction in angiogenesis after ODQ treatment is shown. Data are expressed as means ± S.E.M.; n=23-32; *p< 0.05 vs control. **E.** cGMP-elevating agents increase neo-vascularization in CAM. CAMs were treated with the indicated dose of the NO-activator BAY 41-2272 or the phosphodiesterase 5 inhibitor zaprinast and vessel length determined. Data are expressed as means ± S.E.M.; n=12-36; *p< 0.05 vs control.

Figure 2. ODQ affects endothelial and blood lineage in zebrafish embryos. Images from a compound microscope superimposed: bright field, red [*Tg(gata1:DsRed)*] and green [*Tg(flk1:EGFP)*^{s843}]. Transgenic *Tg(gata1:DsRed);Tg(flk1:EGFP)*^{s843} embryos at 96hpf **(A), (C)** and treated with ODQ

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from 24 to 96hpf **(B, E)**, 10 to 96hpf **(D)** and 48 to 96hpf **(F)**. At 96 hpf, the cranial vessels of ODQ treated embryos (arrowhead in B) are less elaborate than in wild-type embryos **(A)** and there is a cardiac edema (arrow in B). In *Tg(gata1:DsRed);Tg(flk1:EGFP)*^{s843} embryos there are DsRed positive cells circulating throughout the body of the embryo (C and see supplementary movie 1). In ODQ treated embryos from 10-96 hpf there are no *gata1:DsRed* positive cells, the intersegmental vessels are thinner and the posterior cardinal vein (PCV) disorganized **(D)**. When embryos are treated from 24-96hpf few *gata1:DsRed* positive cells can be seen (arrowhead in E and see supplementary movie 2). In embryos treated with ODQ from 48-96 hpf, blood cells accumulate at the posterior part of the embryo. A: atrium; V: ventricle; ISV: intersegmental vessel

Figure 3. sGC activation stimulates the expression of an angiogenic phenotype in EC. A. sGC activation promotes EC proliferation. EC were cultured in the presence of the indicated concentration of BAY 41-2272 and incubated for 48hr. Cell number was determined using a hemocytometer. Data are expressed as means ± S.E.M.; n=12; *p< 0.05 vs control. **B.** sGC activation promotes EC migration. EC were allowed to migrate for 4hr in the presence of BAY 41-2272 (1μM) or 8Br-cGMP (1mM). Data are expressed as means ± S.E.M.; n=4; *p< 0.05 vs control. **C.** Inhibition of sGC blocks tube-like structure formation. HUVEC were cultured on Matrigel® in the presence of ODQ (10μM) or DMSO (control) for 24hr. Network-length was determined using image analysis software. Data are expressed as means ± S.E.M.; n=6; *p< 0.05 vs control. **D.** sGC mediates VEGF-induced cell migration. EC were allowed to migrate for 4hr in the presence of VEGF (50ng/ml) with or without ODQ (10μM). Data are expressed as means ± S.E.M.; n=6; *p< 0.05 vs control, #p<0.05 vs VEGF.

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Figure 4. Adenovirus-mediated overexpression of sGC promotes EC proliferation, migration and tube-like networks formation. A. HUVEC were infected with GFP or sGC-subunit containing viruses. After 24hr cells were plated at $6x10^3$ cells/cm^2 and allowed to proliferate in complete medium for 48hr. Cells were then trypsinized and counted using a hemocytometer. Data are expressed as means ± S.E.M.; n=8; *p<0.05 vs GFP. **B.** Cells infected as in A and after 24-48hr were trypsinized, place in transwells and allowed to migrate for 4hr in the absence or presence of VEGF (50ng/ml). Data are expressed as means ± S.E.M.; n=6; #p< 0.05 vs control, *p<0.05 vs GFP. **C.** Uninfected cells or cells overexpressing GFP or sGC were placed on Matrigel® and allowed to form networks. Alternatively, uninfected cells (hatched bars) were incubated with BAY 41-2272 (1μM). After 24hr cultures were photographed and network area quantified by image analysis software. Data are expressed as means ± S.E.M.; n=7; *p<0.05 vs GFP or DMSO. D: Representative photomicrograph of GFP- or sGC-infected cells grown on Matrigel®.

Fig.5. Increased sGC expression stimulates ERK1/2 and p38 cascades. Representative photomicrographs (B) and quantitation of pERK1/2/total ERK ratios and pp38/p38 ratios (A) in naïve, GFP-infected and sGC-infected cells. Data are expressed as means ± S.E.M.; n=4; *p<0.05 vs GFP. **C&D.** Cells were treated for 2hr with ODQ (10μM), lysates were then extracted and blots incubated with the indicated phospho-specific and total antibodies. Data are expressed as means ± S.E.M.; n=4; *p<0.05 vs control;. #p<0.05 vs α1/β1. **E.** p38 inhibition attenuates BAY 41-2272 and sGC-overexpression-induced EC migration. Cells were infected with a GFP- or a sGC-expressing virus. After 20-48hr cells were incubated with the p38 inhibitor SB203580 (10μM) for 1hr; they were then placed in a transwell and allowed to migrate across a porous membrane for 4hr. Alternatively, naïve cells (hatched bars)

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were stimulated with BAY 41-2272 (1μM) with or without SB203580 pretreatment and allowed to migrated for 4hr. Data are expressed as means ± S.E.M.; n=5; *p< 0.05 vs GFP or DMSO. #p<0.05 vs α1/β1, ¥ p<0.05 vs BAY 41-2272.

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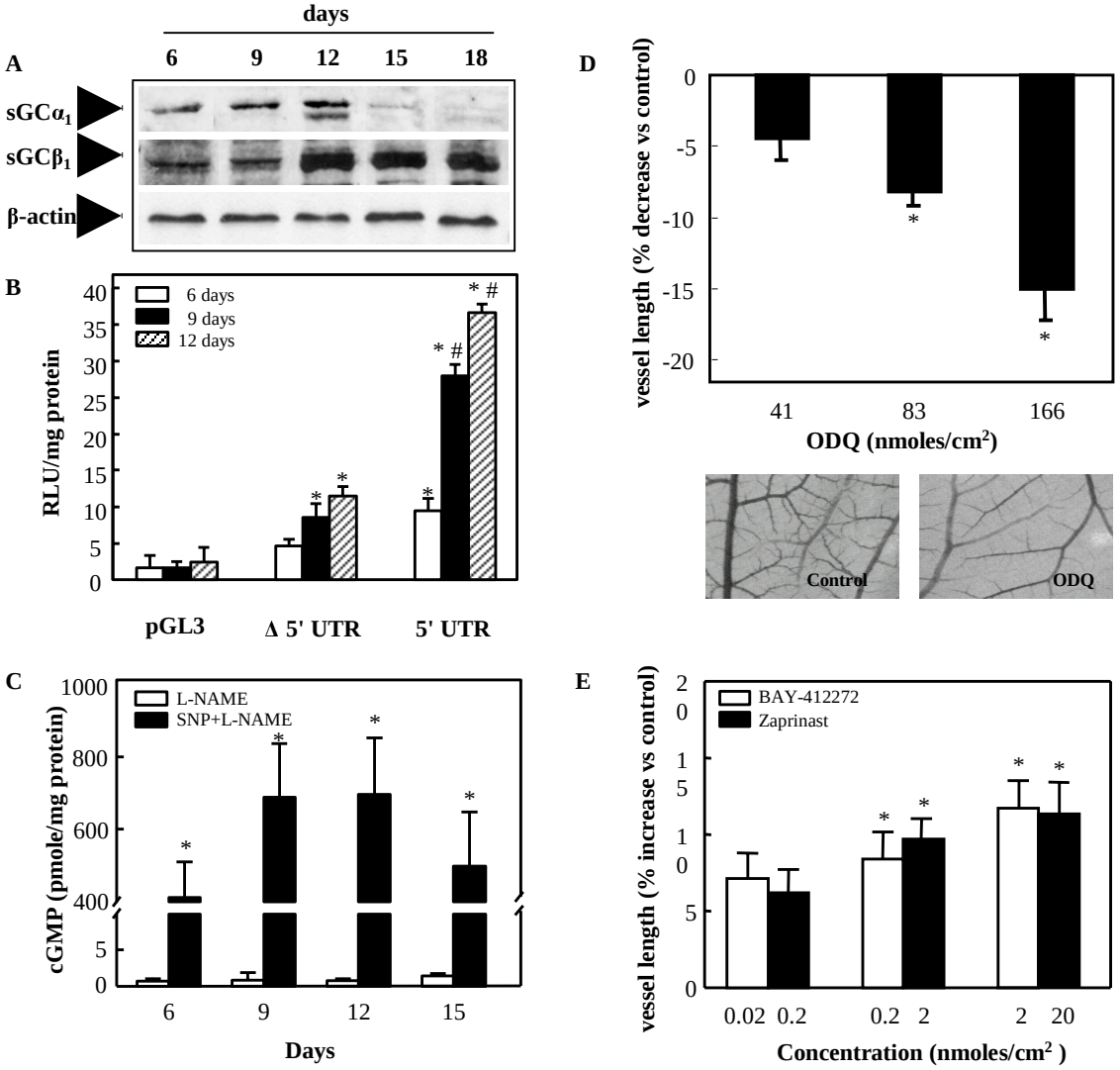

Figure 1

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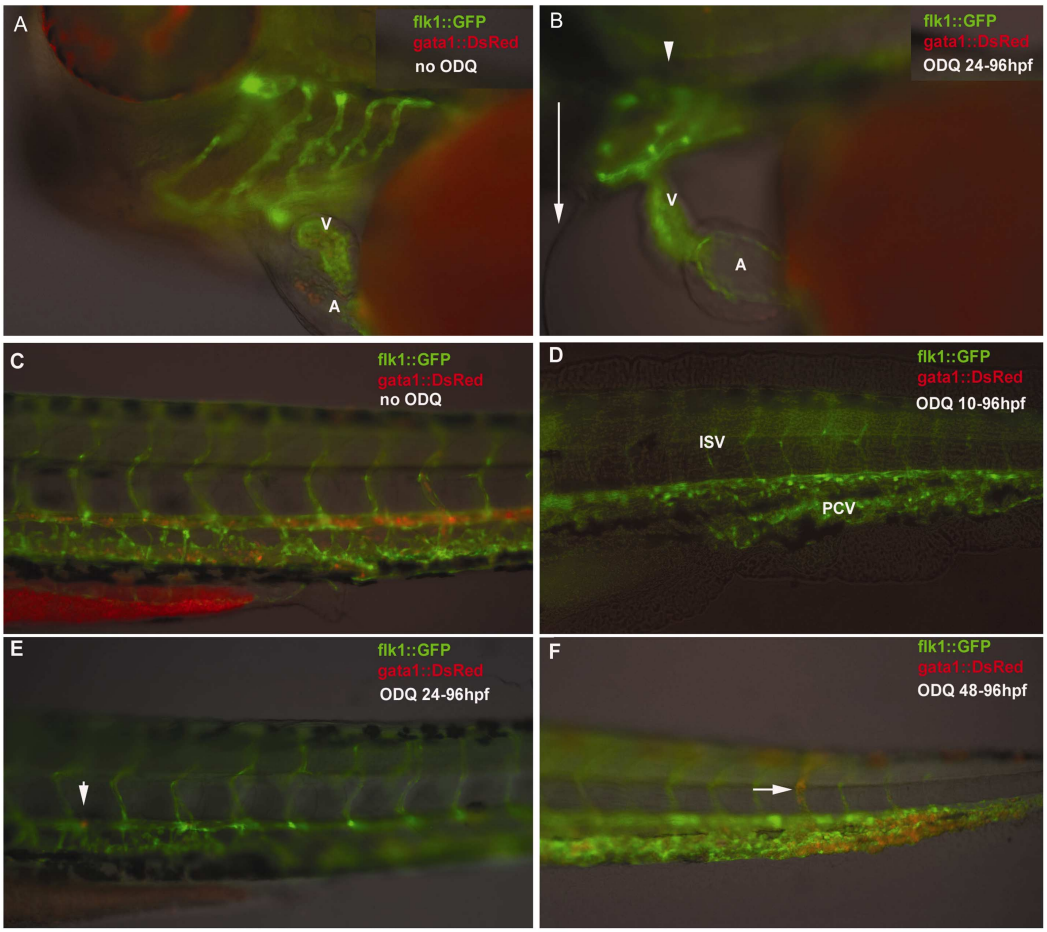

Figure 2

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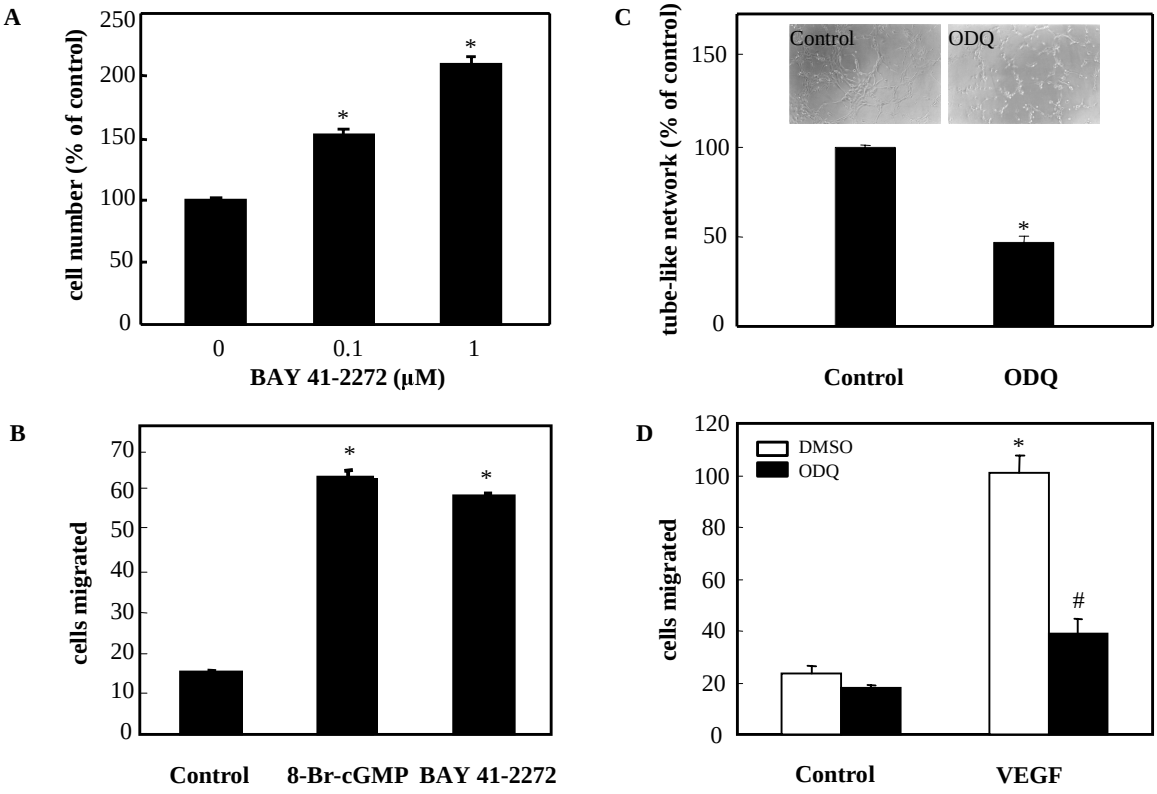

Figure 3

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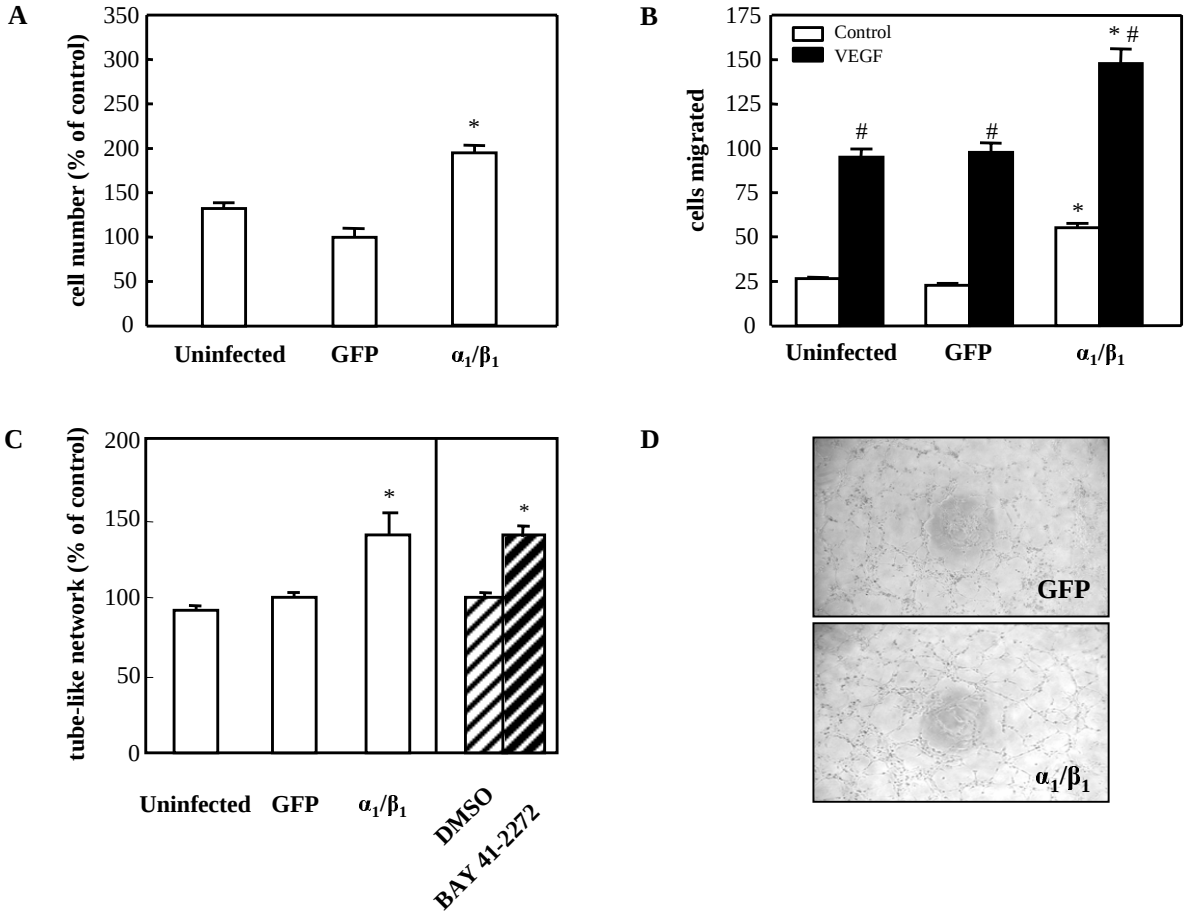

Figure 4

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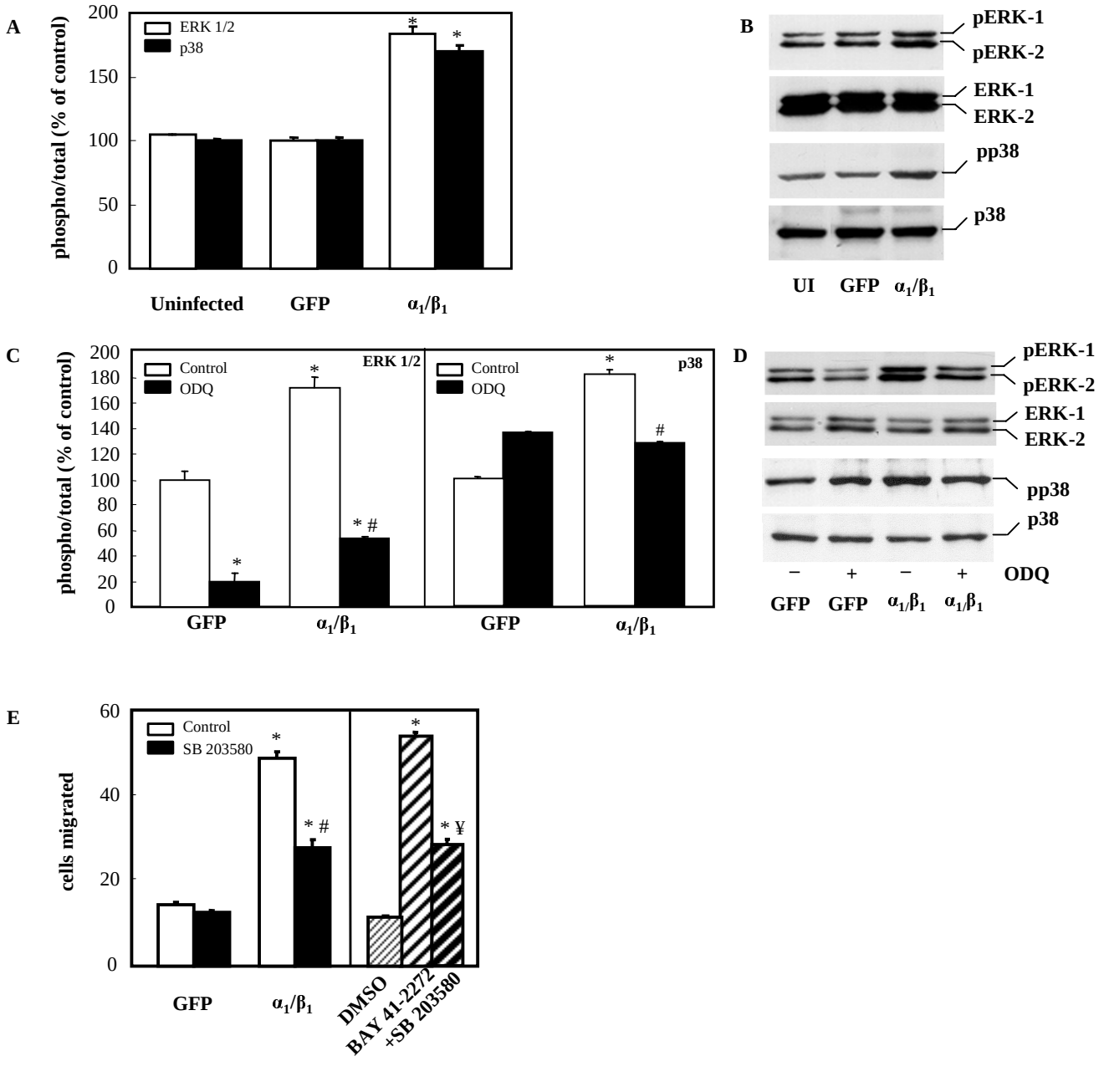

Figure 5

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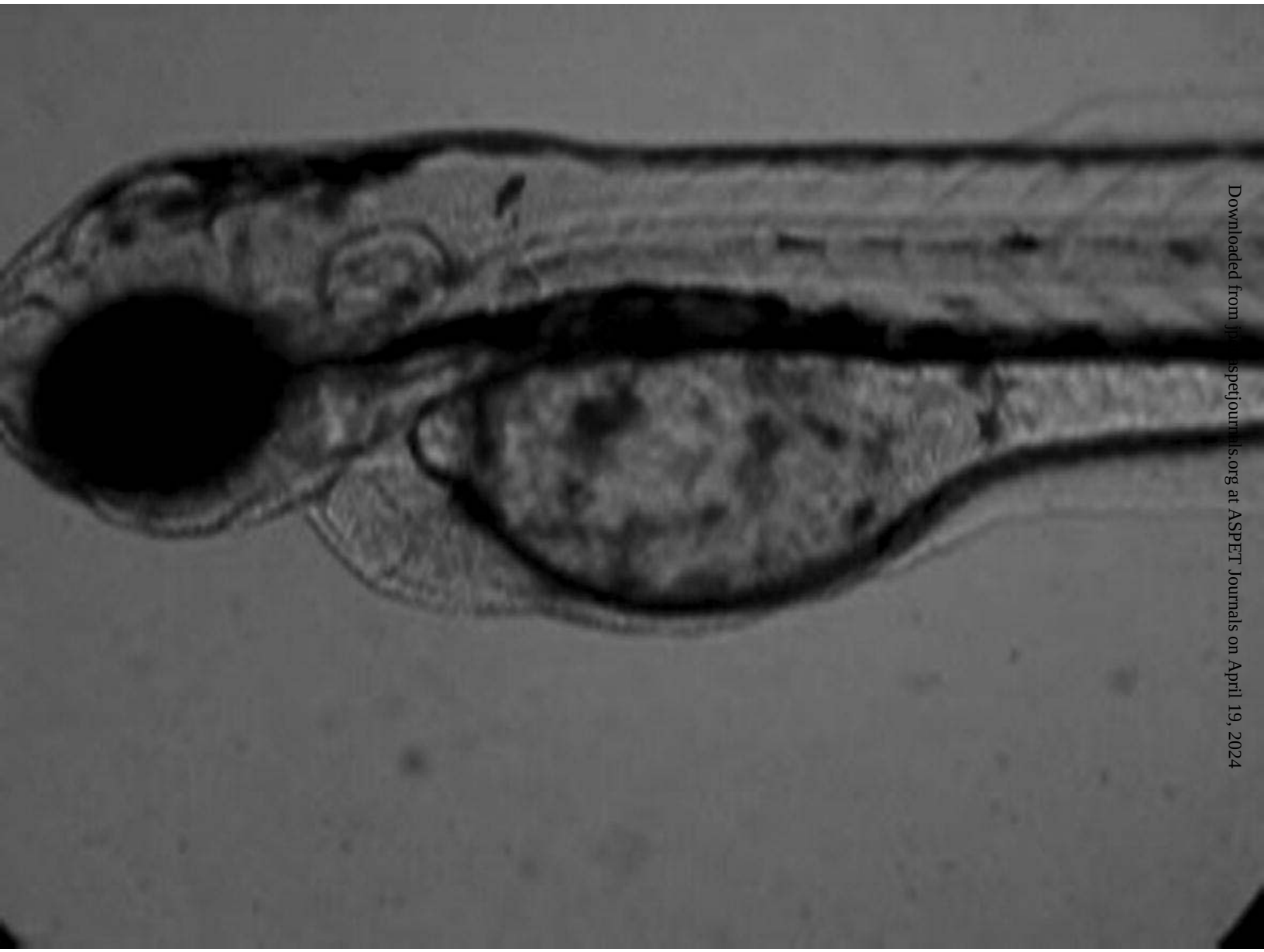

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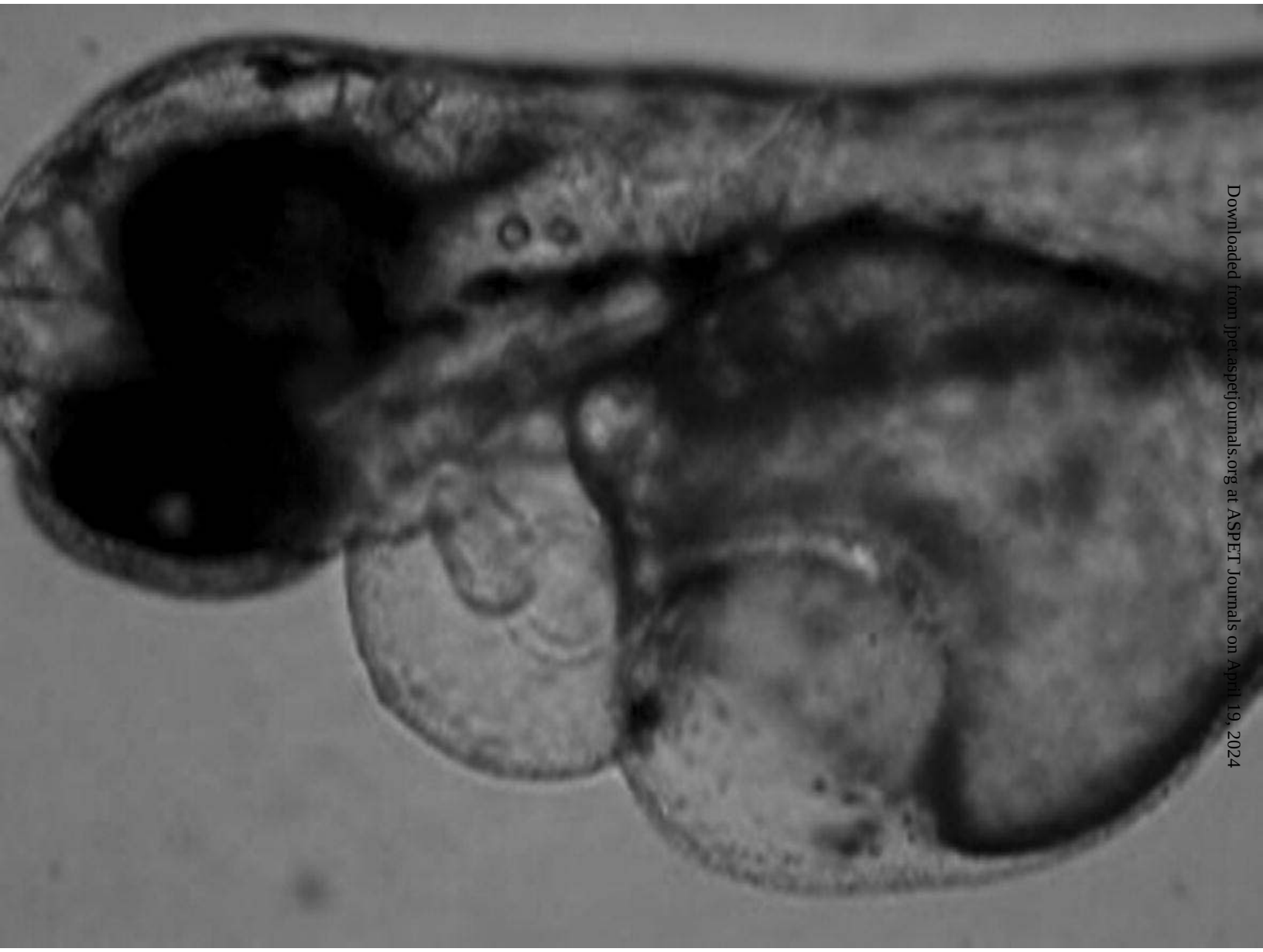

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