Materials and Methods

Reagents, Adenovirus, Apparatus

All cytokines were purchased from Peprotech, Rocky Hill, N , Staurosporine, 1400W were purchased from Enzo Life Sciences, Farmingdale, NY , TW37 was purchased from R&D systems. Adenovirus: shCYGB and GFP control came from Welgen Inc (Worcester MA), ORF of Cytoglobin with C terminal HIS & flag tags and RFP control from ViGene Biosciences Rockville, MD. Hypoxia chambers were purchased from Billups-Rothenberg, Del Mar Ca and used according to manufactures instructions.

Cell culture methods

Rat vascular smooth muscle cells (RVSM) were isolated from the aortas of 5 week old male Sprague-Dawley rats by enzymatic dissociation as previously described [1]. Cultures were maintained in a 1:1 mix of low glucose Dulbecco's Modified Eagle Medium/F12 supplemented with 2 mM L-glutamine, Antibiotic/Antimycotic (100 U/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 µg/ml AmphotericinB) (Mediatech, Manassa, VA) and 10% FBS (Hyclone, Logan UT). Cells were used between passages 1-3.

Human aortic smooth muscle cells (HAoSMC) were obtained from Life Technologies Corporation (Carlsbad, CA) and cultured as directed in their Medium 231 supplemented with fetal bovine serum 4.9% v/v, human basic fibroblast growth factor (hFGF) 2 ng/ml, human epidermal growth factor (hEGF) 0.5 ng/ml, heparin 5 ng/ml, recombinant human insulin-like growth factor-I 2 µg/ml, BSA 0.2 µg/ml(SMGS) and Gentamicin (10µg/ml)/Amphotericin B (0.25µg/ml). Cells were used between passages 4 and 10.

Balloon injury, adenoviral treatment and tissue collection

Animal protocols including surgeries were approved by the Institutional Animal Care and Use Committee at the Albany Medical College. Male Sprague-Dawley rats 375-425 g, (Taconic Farms, Germantown, NY) were anesthetized with 2-3% isoflurane. Following a ventral midline incision in the

neck the muscle, connective and nervous tissues were gently dissected away from the left common carotid artery completely exposing the bifurcation into the internal/external branches. After distal ligation of the external carotid artery, the remaining blood flow was temporarily interrupted with placement of microvascular clamps on the internal branch and the proximal end of the common carotid. An arteriotomy was made in the external branch through which a 2F Fogarty balloon catheter (Edwards Lifesciences, Irvine, CA) was inserted and advanced down the common carotid artery to within a few mm of the clamp. The balloon was inflated to approximately 1.6 atm pressure and retracted slowly with gently rotation to within a few mm from the incision and then deflated, this procedure was repeated three times. After balloon injury, concentrated adenoviral solutions encoding shCygb (50 μl) or noncoding controls (50 μl) were infused into the injured segment of the common carotid artery via a 24-gauge catheter and incubated for 30 minutes. The viral solution remaining in the lumen was removed to avoid dissemination into the systemic circulation. The arteriotomy was tied off with an additional ligature, and the clamps were removed to restore blood flow thru the common carotid artery and its internal branch. The surgical incision was sutured and the rats were given an intramuscular injection of the analgesic Buprenex (0.20 mg/kg) and allowed to recover. At the designated time points the rats were euthanized by asphyxiation in a CO_2 chamber and both the left (injured) and right (control) carotid arteries were harvested for western blot analysis (protein lysates) or snap frozen in disposable plastic cryomolds containing a cryoprotective embedding medium, OCT (Tissue Tek) and stored at -80^{o}C for later cryosectioning and imaging.

Mouse carotid ligation studies

Animal protocols including surgeries were approved by the Institutional Animal Care and Use Committee at the Albany Medical College. Cytoglobin -/- (*Cygb*$^{-/-}$) mice on a C57BL/6 background and there wild-type littermates (*Cygb*$^{+/+}$) were generated as previously described[2]. All experiments were performed in 6 to 8 week-old male *Cygb*$^{-/-}$ and *Cygb*$^{+/+}$ littermates. For the carotid ligation studies, mice were anesthetized with inhaled isoflurane. After midline incision of the neck, complete ligation of the left common carotid artery was performed, just proximal to the carotid bifurcation. The right carotid

artery served as the uninjured control. Aortas, heart, and carotids were harvested at the indicated time and processed for qRT-PCR, Western blot, and histological analysis.

High resolution ultrasound measurements

Ultrasound characterization of the *Cygb*$^{-/-}$ and *Cygb*$^{+/+}$ mice were performed using a VEVO 3100 high resolution (Fujifilm, Visualsonics). Mice were anesthetized with inhaled isoflurane, and a heated stage was used to maintain body temperature. The *M*-mode was used to measure vessel dimensions. The electrocardiogram and respiration were used to gate all measurements.

Hematoxylin/Eosin, Immunofluoresence and TUNEL

7-10 µM tissue sections were cut from prepared OCT blocks using a Leica CM1850 cryostat and transferred to Colorfrost Plus Microscope slides (Thermo Fisher Scientific), and stored at -80^{o}c until use. For H&E: Slides were air dried briefly, fixed in 10% formalin then stained with a progressive H&E staining kit (Cardinal Health). Slides were cover slipped with VectaMount permanent mounting media (Vector Labs Burlingame, CA). For immunofluorescence:, the sections were air dried, fixed with ice cold acetone for 10 minutes then outlined with a liquid blocker super PAP pen (Cardinal Health). Slides were rinsed with PBS/0.1% Triton–X100 (PBS/T), blocked with 0.5% Fish Gelatin (Sigma) for 30 minutes; incubated with primary antibody overnight at 4^{o}C, washed in PBS/T, incubated with 2^{o}Antibody for 1 hr at RT, washed again and then cover slipped with VectaShield Antifade Mounting Medium with DAPI (Vector Labs). The coverslips were sealed with clear nail polish and the slides were stored at 4^{o}C until viewing. Primary antibodies: rabbit polyclonal Anti-Cytoglobin (Proteintech Rosemone, IL) murine monoclonal anti-Calponin (Sigma, St Louis, IL). Secondary antibodies: Alexa Fluor goat anti rabbit 488 and chicken anti mouse 594 (Invitrogen, Eugene,OR). TUNEL assay VasoTACS in situ Apoptosis detection kit as per manufactures directions (Trevigen Gaithersburg, MD).

Protein extraction and Western blotting

Western blotting was performed as previously described(), briefly protein lysates were isolated from cultured cells directly into RIPA buffer (50 mM Tris-HCL(pH 8), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Sodium Deoxycholate, 2 mM EDTA, HALT protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockfield,IL). Human Aorta and Vena Cava protein lysates were purchase from ProSci Inc Poway, CA. All other tissue samples were first minced with fine scissors, then transferred into a soft tissue homogenizing kit (VWR Radnor, PA) and homogenized into RIPA buffer with a Minilys Tissue Homogenizer (Bertran Corp, MD) according to manufactures instructions. Protein concentrations were determined using a BCA Protein assay (Thermo Scientific). Lysates were then heat denatured with either 2 or 4X Laemmli sample buffer (BioRad, Richmond Ca) and equal amounts were loaded onto 4-20% gradient gels (NuSEP Inc. Bogart, GA). After separation the proteins were transferred onto 0.2 µM PVDF membranes (BioRad) and blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat milk (TBST) for 1 hr at RT. The membranes were then probed with primary antibodies for 1 hr at RT or overnight at 4^{o}C, washed 3X in TBST then incubated with the corresponding HRP conjugated secondary antibodies (BioRad). Signals were detected using Clarity ECL substrate (BioRad) on a Fuji Luminescent Image Analyzer- LAS-4000 equipped with MultiGauge software. The following primary antibodies were used: anti-CYGB, anti-CNN1, anti-ACTA2, anti-MYH11 (Protein Tech, Rosemont, IL) anti-Myoglobin(Santa Cruz Biotech, Santa Cruz, CA), anti-nNOS, anti-GAPDH, anti-Caspase antibodies (Cell Signaling, Danvers, MA), anti-ACTB (Sigma, Saint Louis, MO).

Real-time PCR

Total RNA was prepared from cultured cells using TRIzol (Life Technologies) according to manufactures protocol. Tissues were first homogenized in TRIzol using a soft tissue homogenizing kit (VWR) on a Minilys tissue homogenizer (Bertran Corp). cDNA was synthesized using Qiagen's QuantiTect Reverse Transcription Kits. qPCR analysis was conducted using gene-specific primers, and Perfecta SYBR green Mastermix with low ROX (VWR, Radnor PA) on an Agilent Technologies

Stratagene MX3005P instrument equipped with MxPRO software. Oligonucleotide primers were designed using PrimerBLAST (NCBI) and purchased from IDT (Coralville, IA), they are listed below.

Primer sequences for qRT-PCR

GENE	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	PCR size
Human CYGB	TATGTGGGCCCGGCTCTAT	CTCCATGTGCTTGAACTGGC	115
Human Mb	GGCTCTTTAAGGGTCACCCA	GGGGCTTAATCTCTGCCTCA	174
Human RPS18	ATGGGCGGCGGAAAATAGC	TCTTGGTGAGGTCAATGTCTGC	102
Rat CYGB	CTC TCT GGG GTC ATT CTG GA	ATG GCT GTA GAT ACC GC	105
Rat LMOD1	CCA GTC TCT TTG CCT CGT ATC	CAT CCC TGA ACG TGC CTT AT	98
Rat Mb	CAG GAA GTC CTC ATC AGT CTA TTT	CAG GTC CTC TGA ACT CTT CAT C	108
Rat Myh11	ATT CTC AGC CGT GTG AAC AAA GCC	GCT GCT GCA ACT TCT CAT TGG TGT	151
Rat RPS18	AAG TTT CAG CA CAT CCT GCG AGT A	TTG GTG AGG TCA ATG TCT GCT TTC	140
Mouse CYGB	TCG GCC AAG CAG TAC TTC AG	ACC TCC AGA ATG ACC CCA GA	239
Mouse Mb	CTG AAT GTC TGG GGG AAG GT	TTG GGC TAG AGG CTG GAT CT	243
Mouse Rn18s	ATG CGG CGG CGT TAT TCC	GCT ATC AAT CTG TCA ATC CTG TCC	202

Cytotoxicity Detection

Cell cytotoxicity was measured using Takara's LDH Cytotoxicity Detection Kit (MK410) according to the manufactures instructions. Briefly, RVSM cells were seeded into 96 well plates at a density of 10^4/well in a total volume of 200 µL of 10% growth media and allowed to adhere for 48 hrs. Cells were transduced with adenovirus for 24 hrs, prior to treatment with various compounds to block or

stimulate metabolic pathways for an additional 24- 48 hrs. The original culture media was replaced with phenol red/pyruvate free DMEM (SigmaD5030) with 1% FBS. Compounds used to block or stimulate pathways were again included along with those to induce cell death. The plates were returned to the incubator for varying times before being spun down. 100 µl of the cell free culture media was transferred to a 96 well assay plate, 100 µl of reaction mixture was added to each well and plates were incubated for approximately 30 minutes and absorbance was read at 490 nm.

Human sample harvesting, tissue processing and histology and immunofluorescence staining

Human Arteriovenous fistula (AVF) samples were de-identified discarded segments from patients undergoing surgical revision of failed AVFs at Albany Medical College (Institutional Review Board protocol IRB# 3733). AVF Samples were dissected of any adherent fibrous or fatty acid tissue before immediate preparation for histological studies. 7-10 µM tissue sections were cut from prepared OCT blocks using a Leica CM1850 cryostat and transferred to Colorfrost Plus Microscope slides (Thermo Fisher Scientific), and stored at -80^{o}C until use. For H&E staining, slides were air dried briefly, fixed in 10% formalin then stained with a progressive H&E staining kit (Cardinal Health) and the slides were cover slipped with VectaMount permanent mounting media (Vector Labs Burlingame, CA). For immunofluorescence, the sections were air dried, fixed with ice cold acetone for 10 minutes then outlined with a liquid blocker super PAP pen (Cardinal Health). Slides were rinsed with PBS/0.1% Triton–X100 (PBS/T), blocked with 0.5% Fish Gelatin (Sigma) for 30 minutes; incubated with primary antibody overnight at 4^{o}C, washed in PBS/T, incubated with secondary ntibody for 1 hr at RT, washed again and then cover slipped with VectaShield Antifade Mounting Medium with DAPI (Vector Labs). The coverslips were sealed with clear nail polish and the slides were stored at 4^{o}C until viewing. Primary antibodies: rabbit polyclonal Anti-Cytoglobin , Anti-MYH11, Anti Alpha-smooth muscle cell actin(Proteintech Rosemont, IL), murine monoclonal anti-Calponin (Sigma, St Louis, IL). Secondary antibodies: Alexa Fluor goat anti rabbit 488 and chicken anti mouse 594 (Invitrogen, Eugene,OR).

Statistical analysis

Results are expressed as mean ± SEM, and statistical analysis using paired t-tests or one- or two-way ANOVA for two or more than two group comparisons were used. Differences were considered statistically significant when $p < 0.05$.

Reference Cited

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2. Thuy le TT, Morita T, Yoshida K, Wakasa K, Iizuka M, Ogawa T, Mori M, Sekiya Y, Momen S, Motoyama H, Ikeda K, Yoshizato K and Kawada N. Promotion of liver and lung tumorigenesis in DEN-treated cytoglobin-deficient mice. *The American journal of pathology*. 2011;179:1050-60.