Materials and Methods

Mice

Targeted deletion of Mef2c in the endothelium with Tie2-Cre was as we previously described[1]. Inducible, postnatal deletion of Mef2c used the $Mef2c^{flox/flox}$ allele[2] bred with mice carrying Cdh5(PAC)-$CreER^{T2}$ [3,4] to generate Cdh5(PAC)-$CreER^{T2+}$; $Mef2c^{flox/flox}$. To induce deletion in Cdh5(PAC)-$CreER^{T2+}$; $Mef2c^{flox/flox}$ mice, we performed serial intraperitoneal injections of 1mg tamoxifen solution (Sigma-Aldrich T5648, 10 mg/ml prepared in sunflower-seed oil containing 10% Ethanol) every 24 hours for 5 days. We will refer to the tamoxifen injected Cdh5(PAC)-$CreER^{T2+}$; $Mef2c^{flox/flox}$ mice as $Mef2c^{Cdh5EKO}$. Control animals were either tamoxifen injected Cre-negative littermates, or vehicle injected Cre-positive littermates. Recombination specificity was monitored with mice carrying the $Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze}$ (Jackson Lab stock # 007909)[5] bred into either $Mef2c^{Tie2EKO}$ or $Mef2c^{Cdh5EKO}$. The efficiency of recombination was monitored by qRT-PCR. All murine experiments were performed in accordance with approved protocols of the Albany Medical College Institutional Animal Care and Use Committee.

En face preparation and immunofluorescence labeling

En face immunofluorescence was performed as described with modifications[6]. Mice were euthanized with intraperitoneal injection of sodium pentobarbital (0.1mg/g of animal). A whole-body cardiac perfusion was performed by perfusing 0.9% sodium chloride saline followed by fixative (0.2% paraformaldehyde in PBS, pH 7.4) through the left ventricle of the heart after severing the right atrium. Thoracic aorta and/or carotids were isolated and further fixed in 0.2% paraformaldehyde solution at 4 °C for 1.5 hours. Fixed tissues were permeabilized in permeabilization buffer (PBS containing 0.2% Triton X-100) for 1 hour at room temperature and subsequently incubated with blocking buffer (5.5% FBS in permeabilization buffer) for 1 hour at room temperature. Primary antibodies were diluted in staining buffer (2.75% FBS in permeabilization buffer) and incubated with tissue for 16 hours at 4 °C with gentle agitation. Tissues were subsequently washed three times in permeabilization buffer in 30 minute intervals. Secondary antibodies (conjugated with Alexa Fluor 647, Alexa Fluor 594, or Alexa Fluor 488 (ThermoFisher Scientific)) diluted in staining buffer were added after the third wash and incubated at room temperature for 3 hours. 4, 6-diamidino-2-phenylindole (DAPI) and Alexa Fluor phalloidin (ThermoFisher Scientific) were used in combination with the secondary antibodies when specified. Tissues were subsequently washed three times in permeabilization buffer and one time in PBS with 30 minutes for each step. After the PBS wash, vascular tissues were bisected along the direction of flow, and mounted with Prolong Diamond antifade mounting medium (ThermoFisher Scientific). Images were obtained using Zeiss LSM 510 Meta confocal microscope with manufacturer's software. The quantification and 3D rendering were performed with Imaris software (Bitplane). It should be noted that antibodies penetrate poorly into the tunica media whereas DAPI and phalloidin penetrate well. Therefore, antibody labeling was restricted to the endothelium and intima.

Immunofluorescence labeling of frozen sections

Thoracic aortas were harvested, cut into 0.5 cm sections and fixed with 2% PFA for 2 hours at 4°C. Samples were briefly washed in PBS and incubated with agitation in 30% sucrose dissolved in PBS at 4°C overnight until the samples submerged to the bottom. Samples were placed in O.C.T compound (Tissue-Tek) for embedding. Frozen blocks were sliced into 10 µm serial sections. Sections were permeabilized in permeabilization buffer (PBS contains 1% TritonX-100) for 30 minutes and blocking buffer (3% donkey serum in PBS contains 1% TritonX-100) for 30 minutes. Primary antibodies diluted in permeabilization buffer were incubated with the sections overnight at 4°C. Slides were washed 3 times with 15 minutes

intervals and secondary antibodies (conjugated with Alexa Fluor 647, Alexa Fluor 594, or Alexa Fluor 488 (ThermoFisher Scientific)) diluted in permeabilization buffer were added after the third wash and incubated at room temperature for 60 minutes. Slides were washed 3 times in permeabilization buffer with 15 minutes intervals and mounted in Prolong Gold mountant contains 4, 6-diamidino-2-phenylindole (DAPI).

Endothelial RNA isolation from thoracic aorta

RNA from mouse aortic endothelium was isolated using a previously described protocol with minor modification[7,8]. Briefly, mice were euthanized by intraperitoneal injection of sodium pentobarbital and then the vasculature was perfused with saline solution for 2 minutes via the left ventricle after severing the right atrium. The straight portion of the thoracic aorta was isolated and cleared of periadventitial adipose tissue. For $Mef2c^{Tie2EKO}$ experiments, the thoracic aorta was quickly flushed with 300 ml of TRIzol reagent (ThermoFisher Scientific) using a 25G needle. Eluates of the thoracic aortas from three animals with the same genotype were collected into a microcentrifuge tube. Chloroform (0.2X volume) was added to the eluate, vortexed and centrifuged to separate the aqueous phase. Isopropanol (1.25X volume) was subsequently added to the collected aqueous phase and transferred to an RNeasy Plus (Qiagen) column, and total RNA were isolated following the company protocol. For $Mef2c^{Cdh5EKO}$ experiments, the thoracic aorta was quickly flushed with 500 ml of TRIzol reagent (ThermoFisher Scientific) using a 25G needle. Eluate of the thoracic aorta from a single animal was collected into a microcentrifuge tube. Chloroform (0.2X volume) was added to the eluate to separate the aqueous phase. GenElute-LPA linear polyacrylamide (Sigma-Aldrich) (1 ml) was added to the aqueous phase as a neutral RNA-carrier and isopropanol (1.25 X volume) was subsequently added to the collected aqueous phase to precipitate the total RNA. RNA pellets were washed by 75% ethanol twice and reconstitution with DEPC-treated water. RNA quality was validated by spectroscopy using a NanoDrop 2000 (ThermoFisher Scientific).

Carotid ligation

Complete carotid ligation procedure was performed as described previously with minor modifications[9,10]. Briefly, mice (10 to 12 weeks old) were anesthetized with ketamine and xylazine (0.1 and 0.01 mg/g, respectively, intraperitoneally). A midline incision was performed at the neck region, and the left carotid was isolated and completely ligated just proximal to the carotid bifurcation. The right carotid artery served as the uninjured control. Both carotids were harvested 10 days after the ligation and processed for imaging.

Microarray analysis

RNA was isolated 14 days after the start of tamoxifen treatment from the mouse thoracic aortic endothelium as described and was processed for Affymetrix Mouse Transcriptome Array (MTA 1.0). Briefly, 5ng total RNA was converted to fragmented biotinylated cDNA using the standard WT Pico protocol (Affymetrix). The labeled samples were hybridized to Mouse Transcriptome 1.0 Array (Affymetrix), and the arrays were scanned on a GCS3000 7G scanner using standard Affymetrix protocols. Raw CEL files for the $Mef2c^{Cdh5EKO}$ (n=4) or control (n=4) were normalized using Affymetrix Expression Console (build 1.4.1.46) with Signal-Space-Transformation (SST) algorithm and Robust Multichip Average (RMA) with median polish method summarization. Differential gene expression was statistically determined by one-way ANOVA between $Mef2c^{Cdh5EKO}$ and control samples, and the resulting p-values were corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) with Affymetrix Transcriptome Analysis Console (v3.0). Data from these experiments were deposited in the Gene Expression Omnibus (GEO) under accession number GSE97089.

Reverse transcription and qRT-PCR

RNA was reverse transcribed using SuperScript III First-Strand Synthesis System (ThermoFisher Scientific) with random hexamers following manufacturer's protocol. qRT-PCR was performed on a Mx3000P qPCR system (Agilent Technologies) with iQ SYBR Green Supermix (Bio-Rad). mRNA levels were normalized to the level of Hprt, and the relative expression fold-change between experimental and control was calculated with the comparative Ct method described previously[11]. The oligonucleotide primers are listed in Table II.

Cell culture

Human dermal microvascular endothelial cells (HDMECs) were isolated from neonatal foreskin tissues obtained from Albany Medical Center Hospital with Institutional Review Board approval as previously described[12,13]. Briefly, endothelial cells were dispersed following 16 hours of dispase treatment, amplified, and then separated from other cell types by selection with CD31 antibody-coated magnetic beads (ThermoFisher Scientific). Endothelial identity was assessed by evaluation of VE-Cadherin expression and lectin binding, and then cells were cryopreserved and stored in liquid nitrogen. HDMECs were cultured in EGM-2 MV endothelial growth medium (Lonza) and used between passages 5 and 10. For all experimental protocols, HDMECs were seeded at 1 x 10^5 cells/cm^2 and incubated for 48-72 hours to achieve mature monolayers.

In vitro immunofluorescence microscopy

Cells were seeded on 8-well glass culture slides (BD Falcon) precoated with 0.1% gelatin with treatments and harvest times described in figures. Cells were fixed with 4% paraformaldehyde (Affymetrix) in PBS for 30 minutes at 4°C, washed once with ice cold PBS, and processed for immunofluorescence staining at room temperature according to previously described methods with minor modifications[13]. Briefly, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS (PBS-TX) for 15 minutes, then treated with Image-It FX signal enhancer (ThermoFisher Scientific) for 30 minutes, and subsequently blocked with 5.5% FBS containing PBS-TX for 15 minutes. Primary antibodies (described in **Table I**) were incubated for 2 hours at room temperature with gentle agitation. After three washes in PBS-TX, secondary antibodies conjugated with Alexa Fluor 647, Alexa Fluor 594 or Alexa Fluor 488 (ThermoFisher Scientific) were incubated for 1 hour at room temperature. DAPI and Alexa Fluor 488-conjugated phalloidin were used in combination with the secondary antibodies when specified, and also incubated for 1 hour at room temperature. After two washes in PBS-TX and one wash in PBS, slides were mounted using Prolong Gold antifade mounting medium (ThermoFisher Scientific) and #1 glass coverslips. Images were obtained using a Zeiss Axio Observer Z1 inverted microscope and manufacturer's software.

siRNA transfection

Two independent Stealth RNAi™ siRNA duplexes (ThermoFisher Scientific) targeting human MEF2A, MEF2C, or MEF2D mRNA were used to decrease their expression in culture. Individual siRNA sequences are listed in **Table III**. The respective siMEF2 mixes were equal-part mixes of the two siRNAs. Two Stealth RNAi™ siRNA negative control duplexes were used as non-targeting control (Medium GC negative control duplex #2, 12935-112; and #3, 12935-113). siRNA duplexes were delivered into HDMECs at 40 nM final concentration per 1 x 10^5 cells/cm^2 in suspension using Lipofectamine RNAiMax (ThermoFisher Scientific) in Opti-MEM reduced serum media (ThermoFisher Scientific). 10 hours after seeding, an equal volume of EGM-2 MV medium was added. A complete medium change was performed at 24 hours after seeding, replacing with EGM-2 MV medium. The knockdown efficiency of each set of siRNA duplexes was evaluated by qRT-PCR and western blot.

Gel electrophoresis and immunoblotting

Cells grown on 35 mm plates were rinsed with ice-cold PBS and lysed with Laemmli buffer (300 ml) containing complete protease inhibitor mixture (Roche Applied Science) and PhosSTOP phosphatase inhibitor mixture (Roche Applied Science) on ice. Cell lysates were scraped and subsequently boiled for 5 minutes. Cell lysates (30 ml) from each condition were then loaded on standard SDS-PAGE gels and transferred to nitrocellulose membranes. Immunoblots were performed by blocking the membranes with 3% nonfat dry milk in TBS and incubating at 4°C overnight with primary antibodies described in **Table I**. Secondary antibodies conjugated with HRP (Jackson ImmunoResearch Laboratories) were incubated for 2 hours at room temperature. Membranes were developed using SuperSignal West Pico or Femto chemiluminescent substrate (Pierce) and a Fujifilm LAS-3000 imaging system.

Table I. Antibodies and reagents

Primary antibodies and labeling reagents	Company	Cat.# or reference	Used for
VE-Cadherin	Santa Cruz	sc-6458	WB, IF
ZO-1	ThermoFisher	61-7300	IF
β-Actin	Sigma-Aldrich	A5316	WB
GAPDH (71.1)	Sigma-Aldrich	G8795	WB
MLC (MYL2, C-17)	Santa Cruz	sc-34490	WB
di-phospho-MLC (Thr18 and Ser19)		Clements et al. 2005 [14]	WB, IF
MEF2 (C-21)	Santa Cruz	sc-313	WB
MEF2C (D80C1)	Cell Signaling	5030	WB
MEF2D	BD Biosciences	610774	WB
PECAM1	BD Biosciences	550274	IF
MYH11 (BT-562)	Biomedical Technologies	5620313	IF
Acta2 (1A4) conjugated with Cy3	Sigma-Aldrich	C6198	IF
S100A4	EMD Millipore	07-2274	IF
BMP4 (3H2)	EMD Millipore	MAB1049	IF
Fibronectin (FN-3E2)	Sigma-Aldrich	F6140	IF
Alexa Fluor 488-conjugated phalloidin	ThermoFisher Scientific	A12379	IF
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	ThermoFisher Scientific	D1306	IF

Note: WB=Western Blotting, IF=Immunofluorescence

Table II. Mouse oligonucleotide primer for qRT-PCR

Gene	Forward	Reverse
Mef2a	AGACAAGGTGACTGAAAATGGG	CAGAGCACACTGAGTTCATAGG
Mef2c	ATTACGAGGATAATGGATGAGCG	TGTTGGTGCTGTTGAAGATGA
Mef2d	AGACCTCAACAGTGCTAATGG	GGATGACTTTGTTTAGGCTGTTG
Klf2	CGCCGCCACACATACTTG	AACTTCCAGCCGCATCCTT
Klf4	ACTTGTGACTATGCAGGCTG	ACAGTGGTAAGGTTTCTCGC
Nos3	GGCTTACAGAACCCAGGATG	GGGCAAGTTAGGATCAGGTG
Edn1	GTGTCTACTTCTGCCACCTG	CACTGACATCTAACTGCCTGG
Pdgfb	AGACTCCGTAGATGAAGATGGG	TCTTGCACTCGGCGATTAC
Sema3d	GTGTGGGTACATTGATCTCGG	AGGTGCTCATCTGTCATTACTG

Table III. siRNA sequences target human MEF2

siRNA	Sense strand	Anti-sense strand
siMEF2A#1	GGGCAGUUAUCUCAGGGUUCCAAUU	AAUUGGAACCCUGAGAUAACUGCCC
siMEF2A#2	CCGACUGCCUACAACACUGAUUAUU	AAUAAUCAGUGUUGUAGGCAGUCGG
siMEF2C#1	GACCUGUCAUCUCUGUCUGGGUUUA	UAAACCCAGACAGAGAUGACAGGUC
siMEF2C#2	AUGGGAGGAUAUCCAUCAGCCAUUU	AAAUGGCUGAUGGAUAUCCUCCCAU
siMEF2D#1	GGUUUCUGUGGCAACGCCGAGUUUA	UAAACUCGGCGUUGCCACAGAAACC
siMEF2D#2	GCUCUCCUCCUUACCAGCCUUUAGU	ACUAAAGGCUGGUAAGGAGGAGAGC

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