SUPPLEMENTAL MATERIAL

Orai1-mediated I_{CRAC} is essential for neointima formation after vascular injury

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Detailed Methods

Cell culture

Primary rat VSMCs were isolated from aortas of male Sprague-Dawley rats (150g) by enzymatic dispersion as described before[1]. VSMCs were cultured in media containing DMEM (45%), Ham's F12 (45%), and 10% fetal bovine serum (FBS) with L-glutamine and antibiotics.

Antibodies and Reagents

Polyclonal antibody specific for Orai1 was purchased from Alomone (#ACC-062). STIM1 Monoclonal antibody was from BD Biosciences (#610954). STIM1 polyclonal antibody was a gift from Drs. Jonathan Soboloff and Donald Gill (Temple University). Polyclonal antibody specific for CaMKIIδ2 was described earlier[2]. Polyclonal antibodies specific for proliferating cell nuclear antigen (PCNA) protein and green fluorescent protein (GFP) were purchased from Abcam. Monoclonal antibody specific for β-actin was purchased from Sigma. Monoclonal antibody specific for rat Ki67 was purchased from Dako. Rat-specific siRNA sequences were (siOrai1: CCUGUGGCCUGGUGUUUAU; siSTIM1: UAAGGGAAGACCUCAAUUA; siRNA non-targeting control #1 obtained from Dharmacon). Cell culture media and supplies and most of the other reagents were purchased from Fisher Scientific.

Lentiviral Particles

Rat specific Orai1, STIM1 and Luciferase shRNA were cloned in the lentiviral vector pFUGW-GFP[3] using standard protocols and their sequences are as follows:
Orai1: GACCGACAGTTCCAGGAGCTCAACGAGCT
STIM1: GATGATGCCAATGGTGATGTGGATGTGGA

Luciferase: CGTACGCGGAATACTTCGA
Viral particles were generated using standard protocols. PolyJet was used as a transfection reagent (SignaGen) to transfect HEK293FT cells (Invitrogen). Briefly, the lentiviral constructs pCMV-VSVG, pCMV-dR8.2 and pFUGW-GFP-shSTIM1/shOrai1/shLuciferase were co-transfected into a flask of 95% confluent HEK293FT cells. Cell culture media with viral particles were collected at 48h and 72h after transfection and was concentrated using Amicon Ultra-15 filter by centrifugation.

Balloon Injury and Lentiviral Treatment
All Animals protocols, including surgeries were approved by the Institutional Animal Care and Use Committee at the Albany Medical College Animal Resource Facility, which is licensed by the USDA and N.Y.S. Department of Public Health, Division of Laboratories and Research. Male Sprague-Dawley rats (400~450g, Taconic Farms, Germantown, NY) were anesthetized with ketamine (80mg/kg) and xylazine (5mg/kg). Following a midline cervical incision and muscular tissues separation, the left common carotid artery was exposed and blunt dissection was performed alongside the artery by dull forceps to expose the carotid artery bifurcation into the internal/external branches. Blood flow cessation was performed by arterial clamps, with the following steps. A 2F Fogarty balloon catheter (Edwards) was introduced *via* a small arteriotomy in the external carotid artery and advanced to the common carotid artery. The balloon was inflated by 1.6atm pressure and was inserted and withdrawn three times. Sham-operated rats were treated in the same manner except that no balloon was inserted in the vessel. Concentrated lentiviral solutions encoding shOrai1, shSTIM1 or shLuciferase (50μl) were infused into the injured segment of the common carotid artery and incubated for 30 minutes. After the 30min viral treatment, leftover viral solutions in lumen were aspirated and disposed off to avoid dissemination of viral solutions into the systemic circulation. A permanent ligation was placed in the external carotid artery, and then blood flow in the common carotid artery and its internal branch was restored. Following the suturing of incision, rats were treated by the analgesic Buprenex (0.20mg/kg) delivered through intramuscular injection and allowed to recover.

Mice carotid artery ligation modelC57BL/6 male mice (3-4-month old) were anesthetized by intraperitoneal injection of a mixed solution of xylazine (5mg/kg body weight) and ketamine (80mg/kg body weight). The left common carotid artery was dissected and completely ligated near the carotid bifurcation.

Sections, Hematoxylin/Eosin staining, Immunofluorescence and Immunohisto-chemistry
Rats were euthanized at different time points by asphyxiation in a CO_2 chamber and fragments of carotid arteries were placed in a cryoprotective embedding medium OCT then snap frozen in liquid nitrogen. The specimen was then stored at -80ºC or sectioned in a Leica CM3050 cryostat. Sections were treated by pre-cooled acetone for 10 minutes at 4ºC and air dried. Mice with ligated carotids were sacrificed 21days after injury in CO_2 chamber and both the left (ligated) and right (intact, used as a control) carotid arteries were collected and embedded in paraffin. The specimen was then stored at -80ºC or sectioned in a Leica CM3050 cryostat. Sections were treated by pre-cooled acetone for 10 minutes at 4ºC and air dried. Hematoxylin/Eosin (H&E) staining was performed following standard protocols, except that the incubation time with Hematoxylin was increased to 3 minutes and the time with Eosin-Y was decreased to 15 seconds. For Immunofluorescence staining, the sections were fixed with the pre-cooled acetone for 10 minutes at 4^{o}C and rinsed with 1 x PBS. The sections were then incubated in a PBS washing buffer containing 0.1% Triton X-100 for 10 minutes. The sections were incubated for 30 minutes in blocking buffer (1 x PBS/5% goat serum/0.5% fish

gelatin/0.1% Triton X-100). After blocking, the sections were treated overnight with primary antibody diluted in blocking buffer at 4°C. (Rabbit-anti-STIM1 1:50, Rabbit anti-Orai1 1:75, Rabbit-anti-CaMKIIδ2 1:50, Mouse-anti-smooth muscle myosin heavy chain, SM-MHC 1:50; and Mouse anti-Ki67 1:50). The sections were rinsed with washing buffer and then incubated with secondary antibody diluted in blocking buffer containing DAPI for 2 hours at room temperature. 488nm anti-rabbit secondary antibody (Molecular Probes) or 647nm anti-mouse secondary (Molecular Probes) diluted 1:200. Finally, the sections were mounted with anti-fade mounting media (Sigma). The sections were imaged using confocal microscopy at 63x.

For Immunohistochemistry staining, frozen OCT sections were rinsed with 1x PBS 3 times and were fixed using either acetone at -20ºC or 4% paraformaldehyde at room temperature for 10 minutes. Then sections were rinsed with 1 x PBS and endogenous peroxidase was quenched by incubating sections in 0.3% H_2O_2 for 10 minutes followed by rinsing with 1x PBS. The sections were then incubated in blocking buffer (1x PBS/0.1% Tween-20/2% BSA, 5-10%serum) for 30-60 minutes, rinsed with 1x PBS, and incubated with primary antibody (diluted in 1x PBS/0.1%Tween-20/2%BSA) and incubated overnight at 4°C. Primary antibodies dilutions were as follows: anti-Orai, 1:400; anti-STIM1, 1:2000 (Inset in Figure 6, 1:1200); anti-SMα-actin, 1:800.

Secondary antibodies were incubated with sections for 60 minutes at room temperature and sections were labeled by fresh ABC (Avidin/Biotinylated Enzyme Complex) for 30 minutes at room temperature. Substrate DAB (I drop DAB chromagen in 1mL DAB buffer) was added to sections for 2-5 minutes. Hematoxylin staining was performed for 45 seconds. Between every step, sections were rinsed with1x PBS. Paraffin sections were deparaffinized by Xylene for 10 minutes 2 times, rehydrated by ethanol (100%, 95%, 70%), rinsed with dH_2O, and treated by Citra Plus 1X for 20-60 minutes. Then sections were rinsed by 1x PBS and incubated in blocking buffer (1x PBS/0.1% Tween-20/2% BSA, optional 1-2% serum) for 30 minutes. The remaining steps were similar to those used for OCT sections.

Protein Extraction and Western Blotting

The media (and neointima) of the carotid arteries were harvested by cutting open longitudinally and peeling off the adventitia. Tissues were used for protein extraction which was performed on ice. In brief, tissues were cut into small pieces and grinded with a homogenizer in the presence of cell lysis buffer : 10% Phosphatase Inhibitor, 10% Proteinase Inhibitor, and 1% PMSF in RIPA buffer (50mM Tris HCl, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2mM EDTA). After sonication and centrifugation of the cell lysate, proteins were quantified by BCA assay and then loaded in a 10-12% polyacrylamide gel at 10μg per lane. Diluted primary antibodies: Anti-Orai1 1:2000, Anti-STIM1 1:250 (BD biosciences), Anti-PCNA 1:100; Anti-GFP 1:1,000; Anti-CaMKIIδ2 1:1,000 and Anti-β-actin 1:40,000 were incubated overnight at 4ºC, and diluted secondary antibodies (anti-rabbit 1:20,000 or anti-mouse 1:10,000) were incubated for 1 hour at room temperature. Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Photos were taken by Fuji LAS4000 Imaging Station and band densities were quantified using the Image J software (NIH).

Real-time PCR

Experiments were conducted as described previously[1]. In brief, total RNA was extracted from cells using a Qiagen RNeasy Mini Kit following the manufacturer's protocol. cDNA was made from 0.5 μg of RNA reverse transcribed using oligo(dT) primers (Invitrogen, Carlsbad, CA, USA) and SuperScript III reverse transcriptase (Invitrogen). PCR reactions were completed using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The sense and antisense primers targeting rat STIM1, and Orai1 were described earlier[1]. Real-time PCR analysis was performed using a Bio-Rad iCycler and iCycler iQ Optical System Software (Bio-Rad Laboratories). PCR reactions were performed using Bio-Rad iQ SYBR Green

Supermix. The PCR protocol started with 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 54.3°C, and 45 s at 72°C. Quantification was measured as sample fluorescence crossed a predetermined threshold value that was just above the background. Expressions of STIM1, or Orai1 were compared to those of the housekeeping gene rpL32 (Forward: 5'-GCCCAAGATCGTCAAAAAGA; Reverse: 5'-CGATGGCTTTTCGGTTCTTA) and were measured using comparative threshold cycle values as described previously[1].

Ca^{2+} measurements

Ca^{2+} measurements were performed as described previously[1, 4, 5]. Briefly, coverslips with attached cells were mounted in a Teflon chamber and incubated at 37°C for 45 min in culture media containing 4 μM Fura-2/acetoxymethyl ester (Molecular Probes, Eugene, OR, USA). Cells were then washed and bathed in HEPES-buffered saline solution (140 mM NaCl, 1.13 mM $MgCl_2$, 4.7 mM KCl, 2 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH) for at least 10 min before Ca^{2+} measurements were made. For Ca^{2+} measurements, fluorescence images of several cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2; Intracellular Imaging Inc., Cincinnati, OH, USA). Fura-2 fluorescence at an emission wavelength of 510 nm was induced by exciting Fura-2 alternately at 340 and 380 nm. The 340/380 ratio images were obtained on a pixel-by-pixel basis. All experiments were conducted at room temperature.

Whole-cell patch-clamp electrophysiology

VSMCs were used for whole-cell patch-clamp recordings as described previously[1] and have a capacitance of 41.75 ± 11.71 pF. Patch pipettes of 2.5- to 4-MΩ were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL, USA) with a P-97 flaming/brown micropipette puller (Sutter Instrument Company, Novato, CA, USA). Axopatch 200B and Digidata 1440A (Molecular Devices Corp., Sunnyvale, CA, USA) with pCLAMP 10 software were used for data acquisition and analysis. VSMCs were infected with shRNA-encoding lentiviruses for 5 days before recordings. VSMCs were seeded on round coverslips 36 h before experiments. VSMCs were washed with bath solution containing 135 mM Na-methanesulfonate, 10 mM CsCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 20 mM $CaCl_2$, and 10 mM glucose (pH was adjusted to 7.4 with NaOH). Pipette solution contained 145 mM Cs-methanesulfonate, 20 mM Cs-1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 8 mM $MgCl_2$, and 10 mM HEPES (pH adjusted to 7.2 with CsOH). Divalent-free (DVF) bath solution contained 155 mM Na-methanesulfonate, 10 mM *N*-hydroxyethylene-diaminetriacetic acid, 1 mM EDTA, and 10 mM HEPES (pH 7.4, adjusted with HCl). Only cells with tight seals (>10 GΩ) were selected to break in. On obtaining a GΩ seal and break-in, recordings were made from cells with <10 MΩ series resistance. Cells were maintained at a 0 mV holding potential during experiments and subjected to voltage ramps from +100 to −150 mV lasting 250 ms every 2 s. “Reverse” ramps were designed to inhibit Na^+ channels, 10 μM verapamil was included in bath solution to inhibit L-type calcium channels, and 3 μM nimodipine was added to the bath solution to generally stabilize membrane patches and reach better seals.

NFAT-GFP translocation and NFAT luciferase activity assays

Due to the interference of shLuciferase lentivirus with the luciferase assay and that of GFP fluorescence encoded by the lentiviral particles with that of NFAT-GFP, we used siRNA electroporation instead of shRNA lentiviral infection of VSMC. Rat VSMCs were transfected by siRNA against STIM1, Orai1 or non-targeting control using electroporation (sequences provided above). After 3 days, these cells were transfected with pEGFP plasmid encoding nuclear factor for activated T cells (NFAT)-GFP fusion protein (addgene) or co-transfected with pIL-2-Luc (a generous gift from Dr. Fernando Macian) and Renilla-luciferase plasmid (minTK pRL). Twenty-four hours after transfection, cells were treated with 2μM Thapsigargin for 15 minutes. NFAT

nuclear translocation was monitored by the GFP tag under the fluorescence microscope. NFAT activity was measured 24 hours after treatment with thapsigargin for 15 minutes in cell lysates by (firefly-)Luciferase assay using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI), and normalized to Renilla-luciferase activity.

Statistical analysis

Data are expressed as means ± SE, and statistical analysis using One-way ANOVA was done with Origin software (OriginLab, Northampton, MA). *****, ****** and ******* indicates p values < 0.05, 0.01 and 0.001 respectively. Differences were considered significant when $P < 0.05$.

Legends to supplementary figures

Supplementary Figure I: A. Real-time PCR experiment showing that Orai1 knockdown with shRNA has no significant effect on Orai2 and Orai3 mRNA while significantly decreasing Orai1 mRNA levels. **B.** Fura2 Ca^{2+} imaging experiment showing inhibition of SOCE upon Orai1 knockdown and rescue of SOCE with expression of CFP-Orai1 into Orai-silenced VSMCs. Traces are averages of several cells as indicated. **C**. Statistical analysis on 3-4 independent Fura2 experiments similar to **B**.

Supplementary Figure II: IHC using specific anti-GFP antibody as an indication of shOrai1 virus infection of rat left carotid sections (14 day post injury) injured and treated with shOrai1 lentivirus. Sections stained with secondary antibody alone (control) are also shown. Bottom panels are magnified from regions of vessel sections where indicated.

Supplementary Figure III: IF using specific anti-CamKIIδ2 antibody on rat left carotid sections (14 day post injury) injured and treated with shLuciferase, shOrai1 or shSTIM1 lentiviruses (bottom) and corresponding right non-injured non-treated control carotids (top).

Supplementary Figure IV: IF on rat left carotid vessel sections (14 day after injury) balloon-injured and treated with shLuciferase lentivirus (**A,C,E**) or shOrai1 virus (**B, D, F**) and stained with either anti-Orai1 antibody (**C, D**) or secondary antibodies alone (control; **A,B,E,F**)

Supplementary Figure V: A. Fura2 Ca^{2+} imaging experiments in proliferative synthetic rat VSMCs probing for constitutive Ca^{2+} entry activity using the "Ca^{2+}off/ Ca^{2+}" on protocol. Cells are incubated with nominally free Ca^{2+} solution (-Ca^{2+}) and Ca^{2+}-containing solution (2mM; +Ca^{2+}) where indicated. 10 μM ionomycin is added where shown as a control for the Fura2 signal. **B.** Representative whole cell patch clamp experiment in a VSMC cell dialyzed with a pipette solution where Ca^{2+} concentration if buffered to 150nM using BAPTA. No basal current is detected up to 10 min after break-in.

Supplementary Figure VI: A. eYFP-STIM1 expression in proliferative VSMCs (1.5 μg plasmid DNA) shows typical STIM1fibrilar distribution under basal conditions under confocal microscope. Addition of thapsigargin (2 μM) caused redistribution of STIM1 into sustained relatively small puncta (Size of puncta is dependent on STIM1 expression level; not shown). **B.** Co-expression of eYFP-STIM1 (green channel) and CFP-Orai1 (red channel) shows no significant basal co-localization of STIM1/Orai1. Co-localization occurred only after store depletion with thapsigargin. Right hand graphs show co-localization of STIM1 and Orai1 signals in a cross-section of the cell.

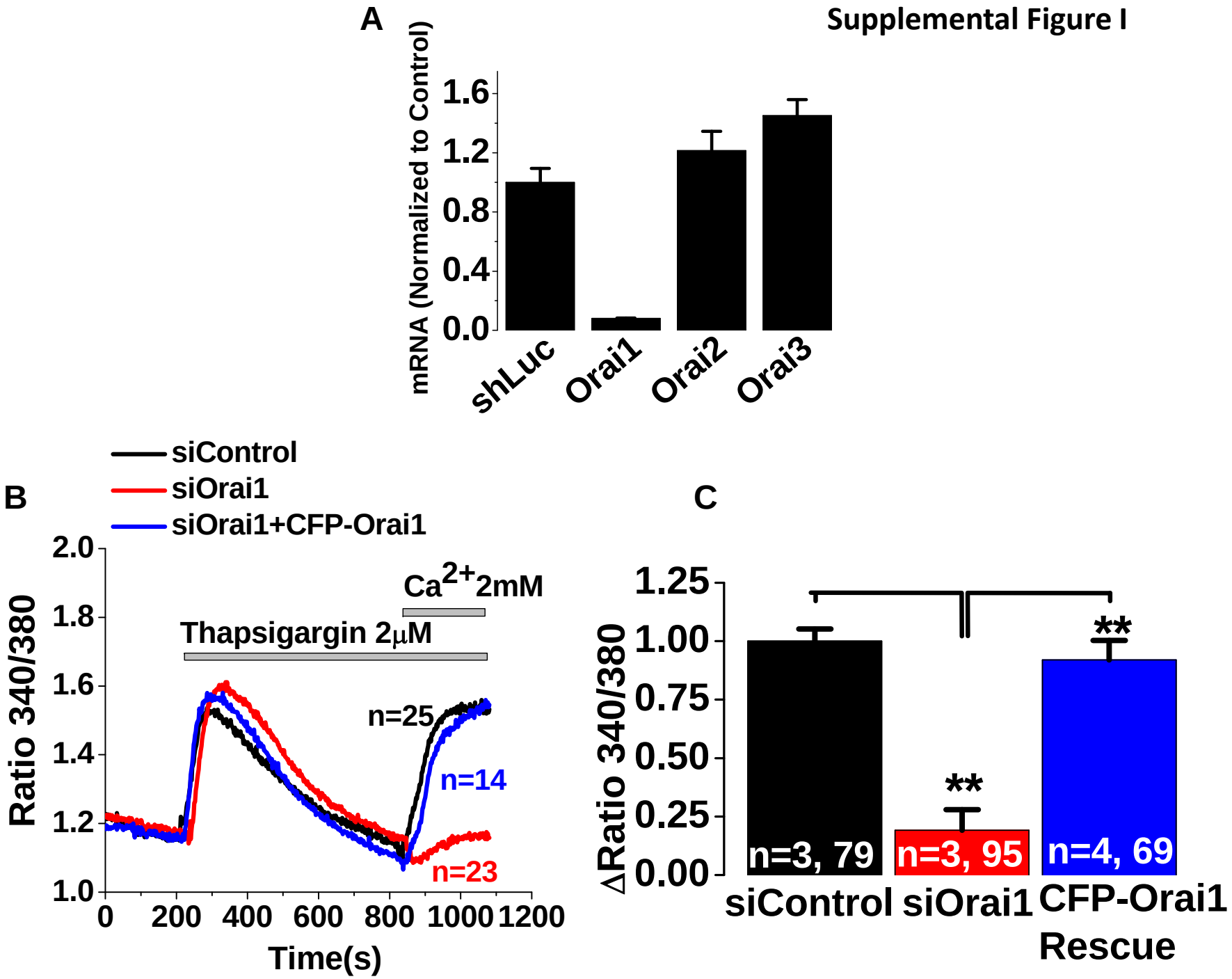
samples	Vessel size (mm^2)		Lumen size (mm^2)	
	R(intact)	L(injured)	R(intact)	L(injured)
shLuc	0.32±0.018	0.40±0.024*	0.24±0.012	0.23±0.015
shOrai1	0.29±0.010	0.35±0.023	0.24±0.008	0.26±0.018
shSTIM1	0.36±0.029	0.35±0.032	0.29±0.022	0.27±0.030

Supplemental Table I: Carotid vessel and lumen size measurements on left injured (L) and right control (R) from rats treated with either shLuc (n=7), shOrai1 (n=5) and shSTIM1 (n=5). * P<0.05 compared to right carotid (R) from shLuc. Lack of significant decrease in lumen size after injury, despite neointima formation, is likely due to compensatory vessel remodeling in response to mechanical injury, as discussed previosuly[6].

References

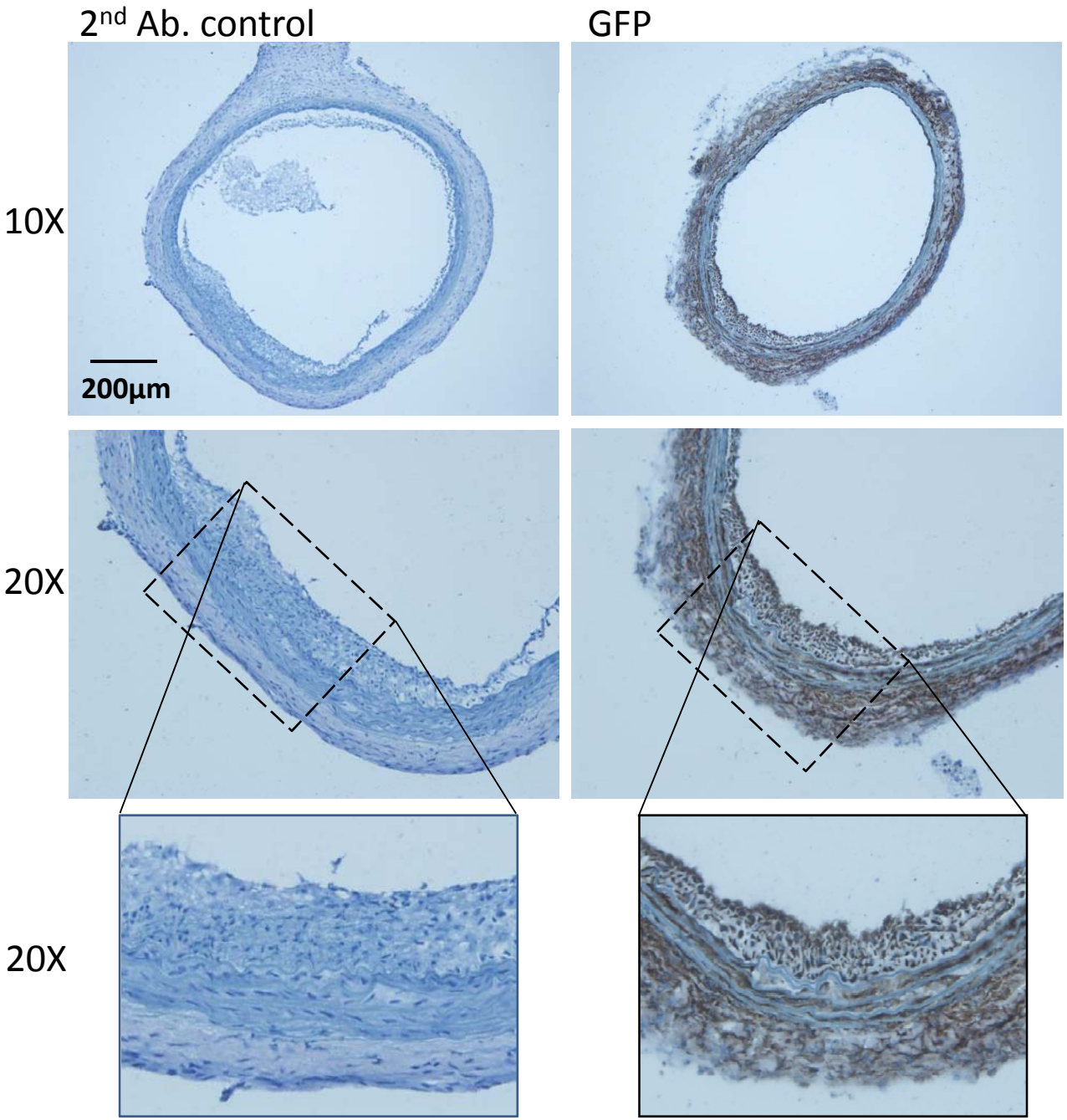
1. Potier M, Gonzalez JC, Motiani RK, Abdullaev IF, Bisaillon JM, Singer HA, Trebak M. Evidence for stim1- and orai1-dependent store-operated calcium influx through icrac in vascular smooth muscle cells: Role in proliferation and migration. *Faseb J*. 2009
2. House SJ, Singer HA. Camkii-delta isoform regulation of neointima formation after vascular injury. *Arterioscler Thromb Vasc Biol*. 2008;28:441-447
3. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science*. 2002;295:868-872
4. Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M. Stim1 and orai1 mediate crac currents and store-operated calcium entry important for endothelial cell proliferation. *Circ Res*. 2008;103:1289-1299
5. Bisaillon JM, Motiani RK, Gonzalez-Cobos JC, Potier M, Halligan KE, Alzawahra WF, Barroso M, Singer HA, Jourd'heuil D, Trebak M. Essential role for stim1/orai1-mediated calcium influx in pdgf-induced smooth muscle migration. *Am J Physiol Cell Physiol*. 2010;298:C993-1005
6. Gibbons GH, Dzau VJ. The emerging concept of vascular remodeling. *N Engl J Med*. 1994;330:1431-1438

Supplemental Figure I



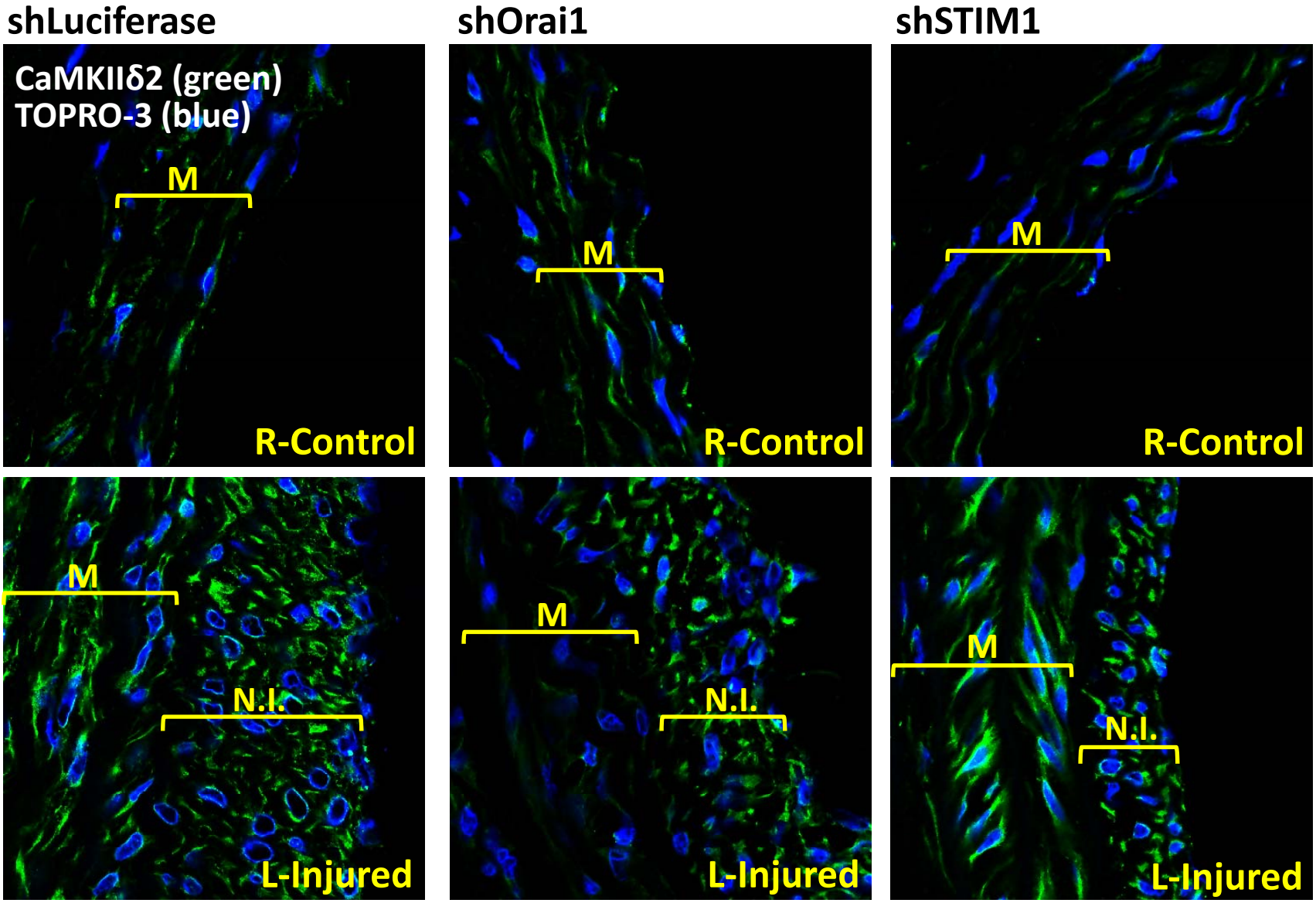
14 days after balloon injury

Supplemental Figure II



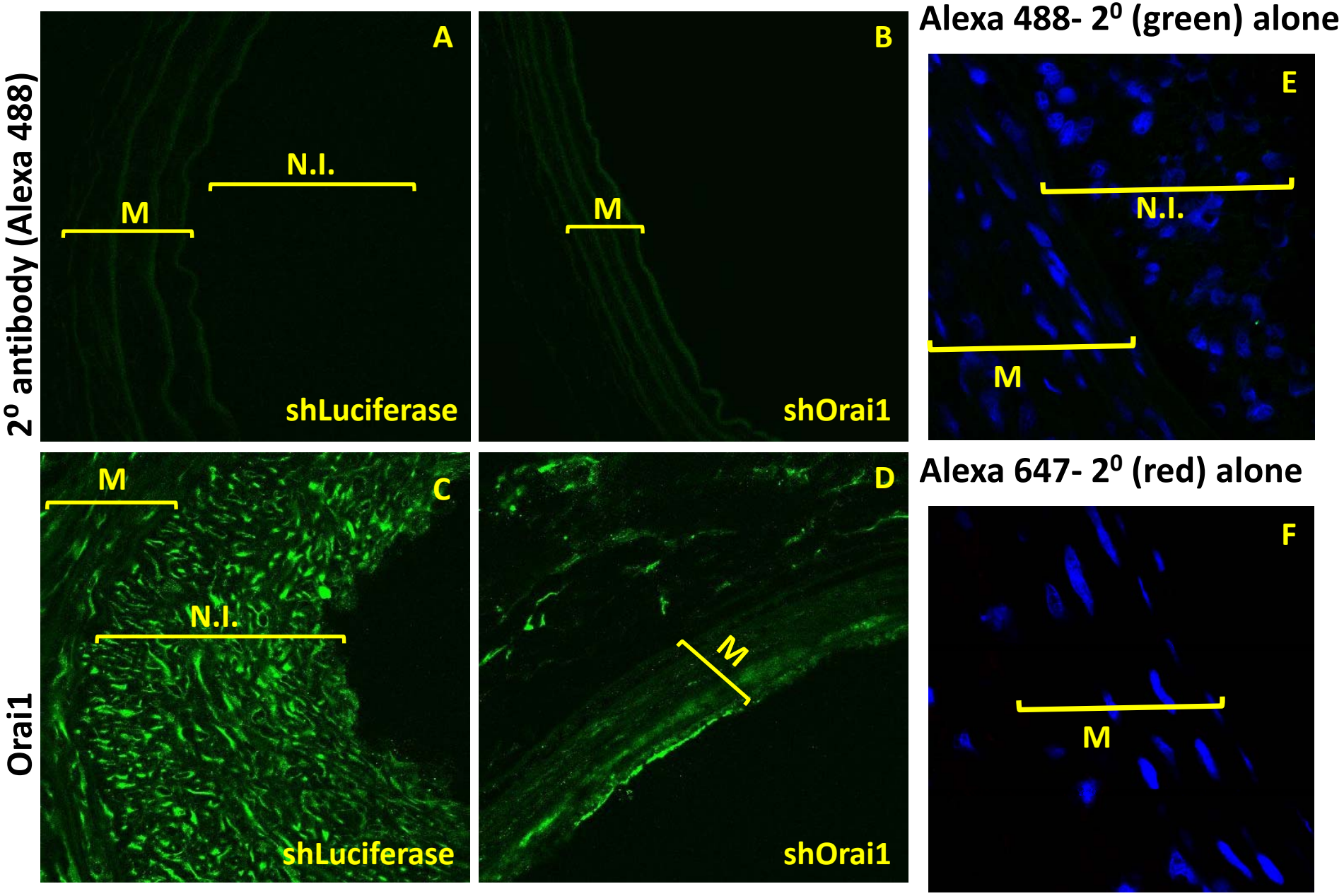
Supplemental Figure III

14 days after balloon injury

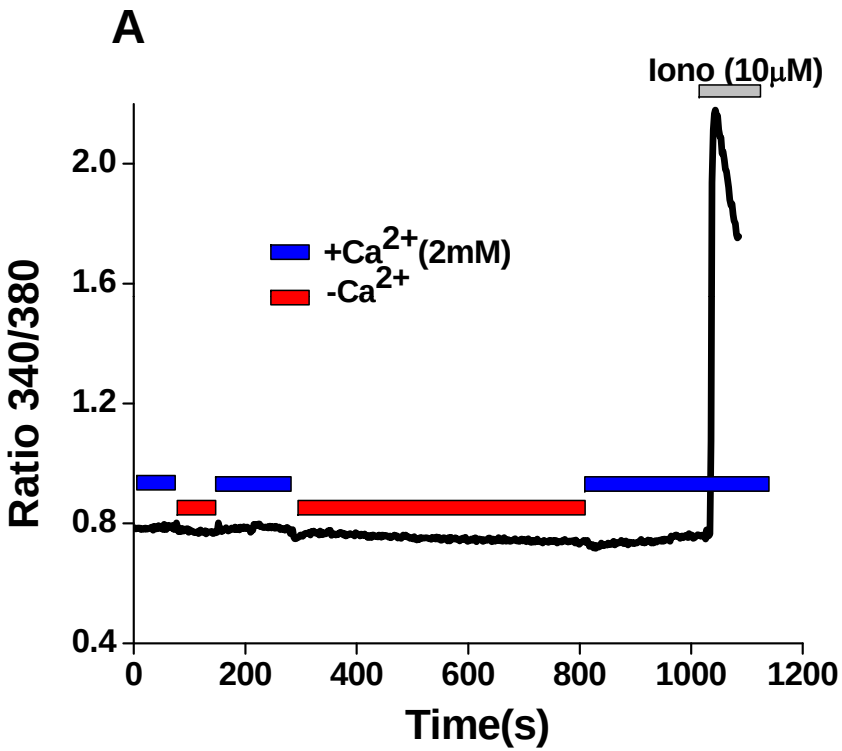


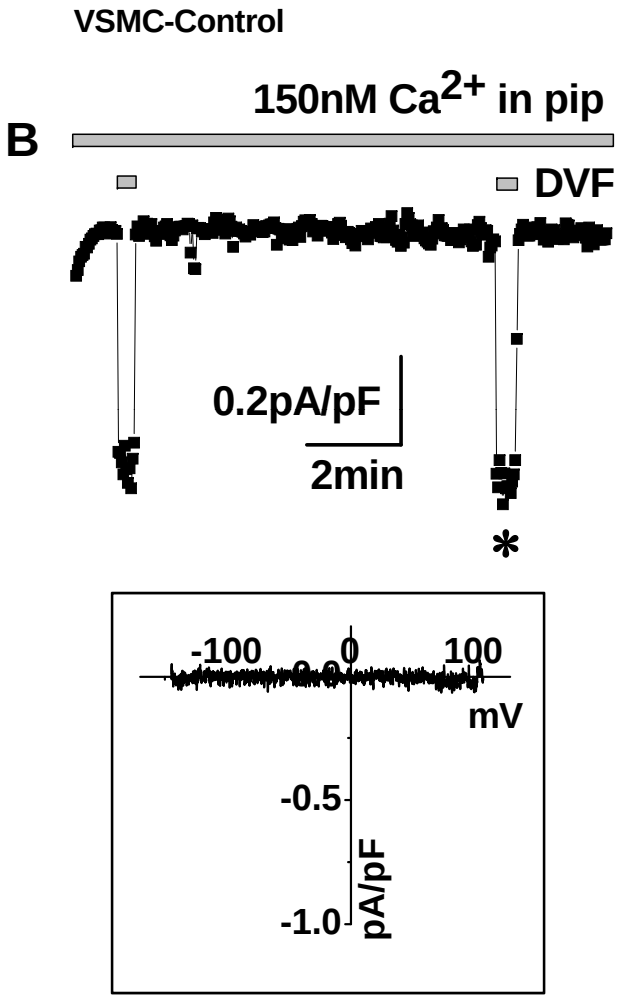
14 days after balloon injury

Supplemental Figure IV



Supplemental Figure V





Supplemental Figure VI