Online Supplement

Materials and Methods

Animals and coronary vessel isolation. Castrated male swine (27-47 kg; 6-8 months old) were initially anesthetized with telazol (5 mg/kg), xylazine (2.25 mg/kg), and atropine (0.05 mg/kg), and maintained under anesthesia during angioplasty by isoflurane inhalation (~2%). Heparin was given with an initial loading dose of 300 U/kg IV, followed by maintenance of 100 U/kg each hour. Swine either remained under isoflurane anesthesia for 2 hours following angioplasty (n=5), or were allowed to recover for 2 days (n=5), 14 days (n=3), or 28 days (n=3). Aspirin was administered orally one day prior to, the day of, and the day following surgery (325 mg), as well as each subsequent day (162 mg) until sacrifice. Swine were euthanized by removal of the heart, which was immediately placed in 4^{o}C physiological saline solution (PSS). Injured and non-injured segments of coronary arteries were isolated and either quickly frozen in liquid nitrogen or placed in paraformaldehyde. Animal protocols were approved by the University of Missouri Animal Care and Use Committee in accordance with the "Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training".

Coronary balloon angioplasty. A 6F HS SH or LCB SH guide catheter (Boston Scientific) was introduced through a 7F sheath, placed in the right femoral artery, and positioned at the left main ostia. Angiograms (Omega Medical) of both the left circumflex (LCX; RAO 40) and left anterior descending (LAD; LAO 30, cranial 30) arteries were obtained with injection of Visipaque contrast medium. Coronary artery diameter was measured using both angiography (Infimed software) and intravascular ultrasound (Volcano; 20 MHz catheter) 2 minutes following nitroglycerine injection (200ug, intracoronary injection via guide catheter). Coronary injury was induced by standard balloon catheter (15-20 mm, Maverick; Boston Scientific) overinflation (1.3-1.4 lumen diameter) three times for 30 seconds each, waiting one minute between inflations. TRAM-34 coated balloons were inflated for 1 minute on the first inflation to allow ample delivery time of TRAM-34 into the vessel wall. Either the LCX or LAD was injured with a non-coated balloon (2 hours n=5, 2 days n=5, 14 days n=3, 28 days n=3), while the remaining artery was injured with a TRAM-34 coated balloon (2 hours n=5, 2 days n=5, 14 days n=3, 28 days n=3). Non-injured segments (10 mm) of both LAD and LCX were used for control. One pig from the 14 day recovery group died of unknown causes following angioplasty

Balloon coating. TRAM-34 (20mg/mL) was dissolved in acetone the day of injury. Balloons were chosen based on vessel diameter and inflated to 6 atm (nominal pressure). The balloon was dipped in TRAM-34 solution for 10 seconds and dried for 1 minute. This cycle was repeated 3 times and followed by a final 5 minute drying time. The balloon was then carefully deflated and immediately guided to the coronary artery for balloon injury.

Immunohistochemistry. Paraformaldehyde fixed, paraffin embedded, 8μm sections of coronary arteries were incubated overnight with primary antibodies against $K_{Ca}3.1$ (Chemicon International, 1:600), REST (1:50,000 [1]), and SMMHC (Biomedical Technologies Incorporated, 1:800), as well as Ki-67 (a marker of proliferating cells, Zymed, 1:200). Sections were then exposed to secondary antibodies for 30 minutes: goat anti-rabbit Ig/HRP (Dako, 1:50, for $K_{Ca}3.1$), goat anti-mouse Ig/AP (Dako, 1:20, for Ki-67), or universal LSAB (for REST and SMMHC). Chromagens included HRP with AEC+ (Vector), AP Blue (Vector), and DAB (Dako).

Analysis of restenosis. All vessel measurements were performed on Verhoff van Giesen (VVG) stained sections of coronary arteries using Image J software (NIH). The neointimal to medial thickness ratio was calculated by dividing the measurement of the thickest portion of the neointima by the measurement of the thinnest portion of the media. The rupture index (RI) was calculated by dividing the length of the internal elastic lamina (IEL) rupture by the length of the intact IEL [2]. Data are presented as the intimal to medial thickness ratio (IMT) normalized to the RI or IMT/RI.

Laser capture microdissection. Laser capture microdissection was performed as previously described [3]. Frozen sections of porcine LCX and LAD (8μm) were stained with Hematoxylin as modified from a previous study [4]. Staining took approximately 1 hour and was performed on a cold plate to minimize RNA degradation. Medial cells, all cells residing between the internal and external elastic laminas, were collected using the Pixcel IIe (Arcturus) system. Medial portions from 6-10 serial sections were pooled into one sample to ensure sufficient RNA yield. RNA was extracted with the Picopure RNA isolation kit (Arcturus) using the manufacturers protocol.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR was performed as described previously [3-5]. cDNA was reverse transcribed from RNA using Superscript III reverse transcriptase kit (Invitrogen). A minus reverse transcriptase reaction was performed to ensure no

genomic DNA contamination. qRT-PCR was performed on the MJ minicycler (Bio-Rad), and data analyzed using Opticon Monitor 3 software (Bio-Rad). Each 25µL reaction contained 1X Syber Green Master Mix (Qiagen), 0.8µM forward and reverse primers (IDT), and 1µg of cDNA. Reactions were initiated by a 95°C hold for 10 min, in order to activate heat stable Taq. Reaction conditions were optimized for each set of primers. K_{Ca}3.1, SMMHC, myocardin, and 18S ribosomal RNA primer sequences were published previously [3]. REST, c-jun, and c-fos primer sequences are given in Table 1. Target gene expression was normalized to 18S using the $2^{-\Delta\Delta CT}$ method [6]. Linearity and efficiency of each PCR condition was verified by creating a standard curve of the critical threshold versus log of the dilution of cDNA.

Detection of TRAM-34 in the vessel wall. Samples collected from the LCX, LAD or right coronary artery (RCA), were cut into pieces, homogenized in 1 mL of acetonitrile/water 1:1, with a Brinkman Homogenizer PT 10/35 for 30 seconds 3 times ensuring that the generator shaft was cleaned thoroughly after each homogenization. The samples were further purified using C18 solid phase extraction cartridges. Eluted fractions corresponding to TRAM-34 were dried under nitrogen and redissolved in acetonitrile. LC/MS analysis was performed with a Hewlett-Packard 1100 series HPLC stack equipped with a Merck KGaA RT 250-4 LiChrosorb RP-18 column interfaced to a Finnigan LCQ Classic MS. Mobile phase: acetonitrile/water with 0.2% formic acid, flow rate 1.0 mL/min, gradient ramped from 20/80 to 70/30 in 5 min., then to 80/20 over 11 min. With the column maintained at 30°C, TRAM-34 eluted at 14.4 min. and was detected by a variable wavelength detector set to 190 nm and the MS in series. Using electrospray ionization MS (capillary temperature 270°C, capillary voltage 1V, tube lens offset -15V, positive ion mode) TRAM-34 was quantified by its base peak of 277 m/z (2-chlorotrityl fragment) and concentrations calculated with a 5-point calibration curve. The related compound TRAM-46 (base peak of 261 m/z, 2-fluorotrityl fragment) was used as an internal standard.

Histological Analysis. Sections were photographed with an Olympus BX40 photomicroscope and Spot Insight Color camera (Diagnostic Instruments). The area of positive staining for SMMHC and K_{Ca}3.1 was calculated as a percentage of total section area utilizing ImagePro Plus (Media Cybernetics). Segmentation parameters were held constant for all samples (R = 220 for K_{Ca}3.1 and R=210, G=160, B=170 for SMMHC). For K_{Ca}3.1 and SMMHC, the % positive area was determined by dividing the mean

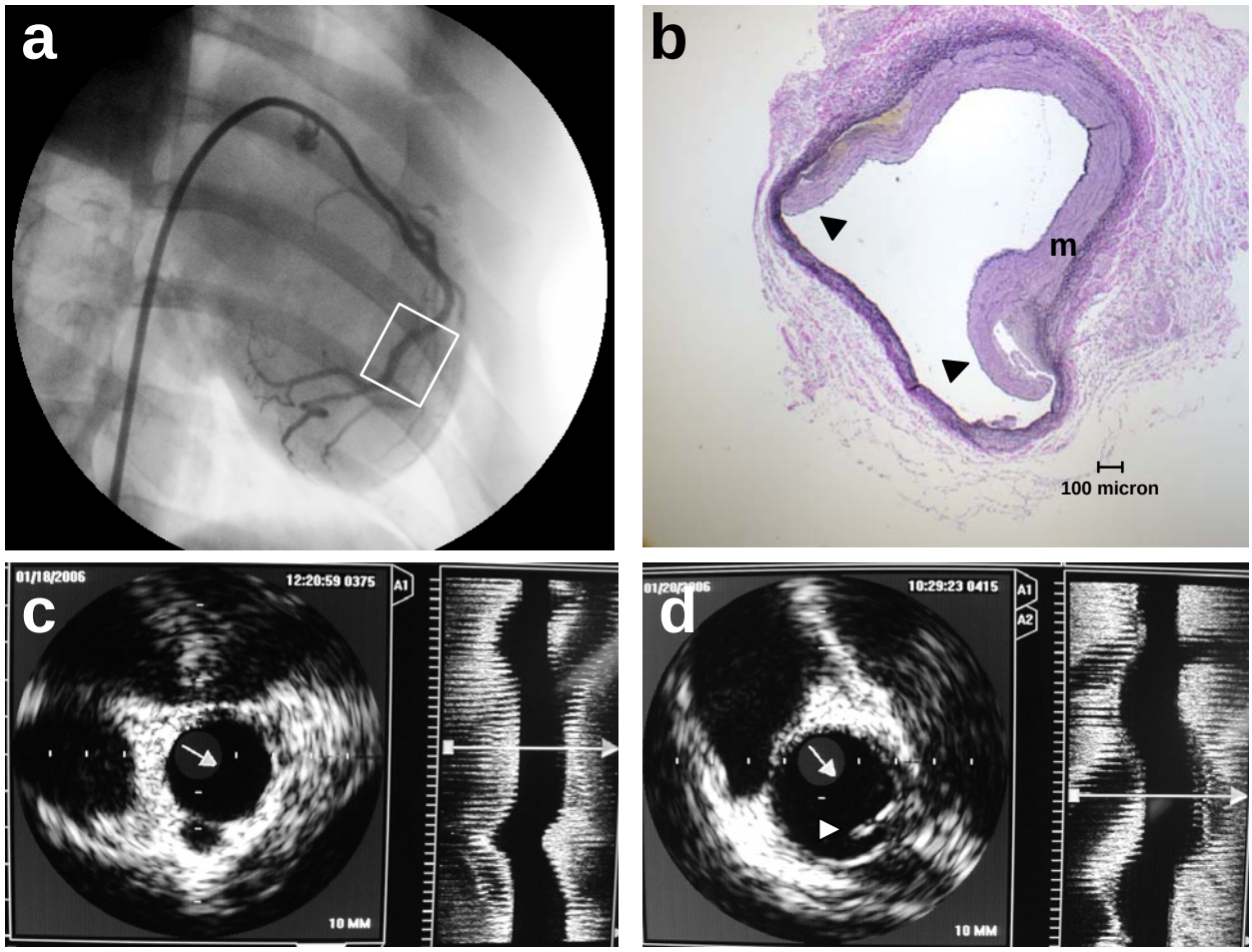
positive area from the total area. For REST, the number of brown (positive) nuclei (manually selected) and total nuclei (blue plus brown) were counted and presented as % positive nuclei (brown/total x 100). Regions of interest were only assessed near the medial tear for both injured and TRAM injured sections. For morphometric analysis, Image J software was used to calculate the intimal to medial thickness ratio (IMT) and the rupture index (RI). IMT was determined by dividing the thickest portion of the intima by the thinnest portion of the media. RI was calculated by dividing the distance between the two torn edges of the internal elastic lamina (IEL) by the distance of the intact (IEL). Data is presented as IMT/RI.

Table I: Primer Sequences (5' to 3')

Target Gene (Accession No.)	Forward Primer	Reverse Primer
REST* (NM_005612)	ATGGCCCTGCCTAACGACATGTAT	TTCACCGACCAGGTAATCACAGCA
c-jun (NM_213880)	CCCAAGATCCTGAAGCAGAG	GATGTGCCCGTTACTGGACT
c-fos (AJ132510)	TTGGGAAGGGAGAGACCTTT	GTTGGGATGAAGTTGGCACT

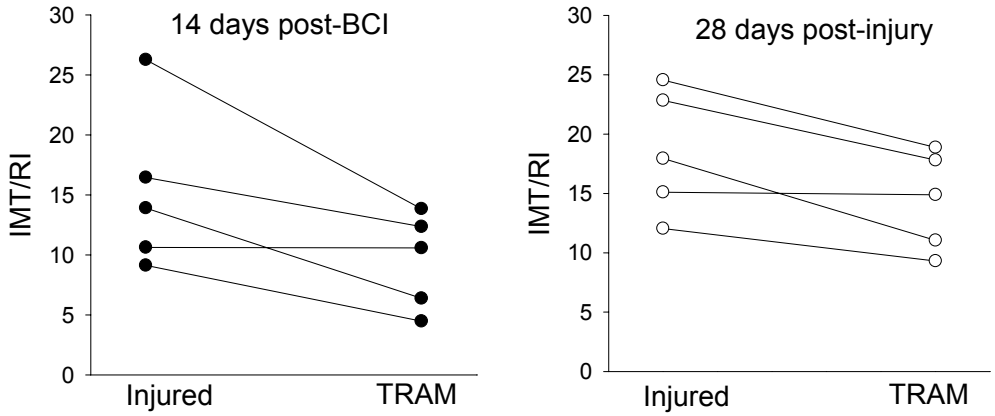
* repressor element-1 silencing transcription factor

Supplement Figure I



Supplement Figure I. Representative angiography and intravascular ultrasound (IVUS) images of coronary balloon angioplasty. Balloon injury was induced by a 35-40% overinflation of either an uncoated or a TRAM-34 coated (20 mg/mL) balloon. Angiogram (a) and IVUS (c, d) images were collected pre- and post-angioplasty. The white box denotes the injured segment of the vessel (a). We observed an increase in vessel diameter immediately following balloon overinflation (d), and as reported by others using this model [7-10] consistently observed a medial tear following injury (b; black arrowheads). In this particular animal, IVUS 2 days following balloon injury demonstrated a medial flap (d; white arrowhead) produced due to medial separation from the external elastic lamina (EEL). Vessel sections were stained with Verhoff van Giesen stain (VVG) to visualize elastin (b; m = media).

Supplement Figure II



Supplement Figure II. Individual injury responses (IMT/RI) for TRAM vs.non-coated arteries in the same animal at 14 and 28 days post-angioplasty. Compared to non-coated balloons (injured), the IMT/RI was consistently reduced (4 out of 5 animals at each time point) by TRAM-34 coated balloons (TRAM).

References

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