“Supplemental Material”

MATERIALS AND METHODS

Cell culture and reagents. Primary rat aortic SMCs grown in a 50:50 mixture of DMEM and F12 media supplemented with 10% fetal bovine serum and the antibiotics penicillin and streptomycin. Cells were growth arrested at confluency for 3 days to induce the expression of smooth muscle contractile genes and promote a differentiated phenotype. To test for the effect of matrix composition, cell culture plates were coated with Coll I (20 µg/ml; Sigma) or Coll IV (20 µg/ml; Chemicon and Trevigen) overnight at 4°C. Plates were then washed with serum free media, and rat aortic SMCs were plated in serum free media at near confluence (3×10^4 cells/cm^2) for the indicated time depending upon the experimental readout. In some experiments, the inhibitors SN50 (Axxora, 30 µg/ml), cyclosporine A (Sigma, 10 µM), or A-285222 (a gift from Abbott Pharmaceuticals, 10 µM) were given for 1 hour prior to experiment initiation to inhibit NF-κB and NFAT signaling, respectively.

Luciferase Assays. SMCs were transfected (FuGene 6) with promoter-reporter (luciferase) constructs at 70% confluency containing the smooth muscle myosin heavy chain promoter (SMMHC-luc), SM alpha actin promoter (SMaA-luc), VCAM-1 promoter (VCAM-1-luc), VCAM-1 promoter with a mutated NFkB/NFAT site (mutVCAM-1-luc), NFATc binding elements (NFATc-luc, Conetech), or SMA promoter containing point mutations in the CArG A, CArG B, or intronic CArG sequence. SMCs were grown to confluency on plastic and then growth arrested for 24-36 hrs. SMCs were then trypsinized and replated onto Coll I or Coll IV for the denoted time and luciferase assay performed.

Quantitative RT-PCR. Total RNA was extracted with TRIzol reagent (Invitrogen), and cDNAs were produced with the BioRad iScript cDNA Synthesis kit per manufacturer’s instructions. To quantify the mRNA, real-time PCR was performed using a BioRad iCycler and the Sybr green visualization dye. All results were normalized to 18S and are expressed as a ratio of target mRNA to 18S mRNA.

Western blotting. Cell lysis and immunoblotting were performed to measure protein expression and posttranslational modification. Briefly, cells were lysed directly in SDS Sample buffer, separated by SDS-PAGE gels, and transferred to a PVDF membrane. The membrane were analyzed by western blotting with mouse anti-SM α-actin (Sigma; 1:5000), goat anti-VCAM (Santa Cruz Biotechnology, 1:500), and rabbit anti-ERK (Santa Cruz Biotechnology; 1:5000). Resulting bands were scanned to tiff files, and band intensity was determined using ImageJ.

Chromatin Immunoprecipitation. Real-time PCR was performed on 1 ng genomic DNA from ChIP. Real-time PCR primers were designed to flank the 5'-CArG elements of SMα-actin and SMMHC, the c-fos CArG. Quantification of protein:DNA interaction/enrichment was determined by the following $2^{Ct(\text{Ref})-Ct(\text{IP})}-2^{Ct(\text{Ref})-Ct(\text{No antibody control})}$.

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Primer sequences were as follows: *SM α-actin* 5-agcagaacagaggaatgcagtggaagagac-3., 5-cctcccactcgcctcccaaacaaggagc-3; *SMMHC* 5.-ctgcgcgggaccatatttagtcagggggag-3, 5-ctgggcgggagacaacccaaaaaggccagg-3

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SUPPLEMENTAL DATA

Figure S1. NF-κB localization and phosphorylation are not differentially regulated by Coll 1 and Coll IV. (A) SMC plating times on Coll I or Coll IV were varied at 24h, 48h, or 5 days, and phosphorylation of the NF-κB p65 subunit was determined using a phosphorylation site-specific antibody. A representative image is shown. n = 3. (B) SMC plating times on Coll I or Coll IV were varied at 2h, 6h, 12h, 24h, 48h, or 5 days. Cells were fixed in formaldehyde and nuclear translocation of the NF-κB p65 subunit was determined by immunocytochemistry. Representative images are shown. n = 3.

Figure S2. SN50 reduces IL-1β-induced VCAM-1 expression. SMCs were plated onto Coll IV and some cells were treated with the NF-κB inhibitor SN50 for 1 hour. Cells were then treated for 24 hours with IL-1β (5 μg/ml). VCAM-1 expression was assessed by Western Blotting as previously described. n = 3 duplicate experiments. * $p < 0.001$.

Figure S3. Coll I and Coll IV do not differentially regulate p38. SMC plating times on Coll I or Coll IV were varied at 24h, 48h, or 5 days, and phosphorylation of p38 was determined using a phosphorylation site-specific antibody. A representative image is shown. n = 3.

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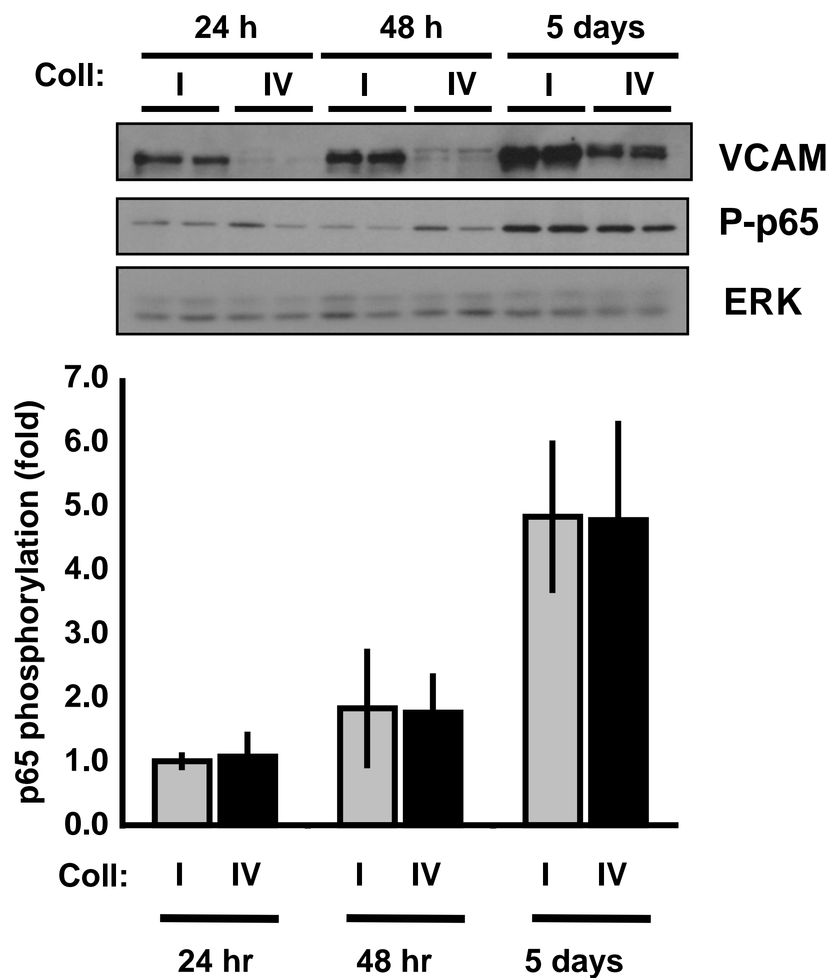
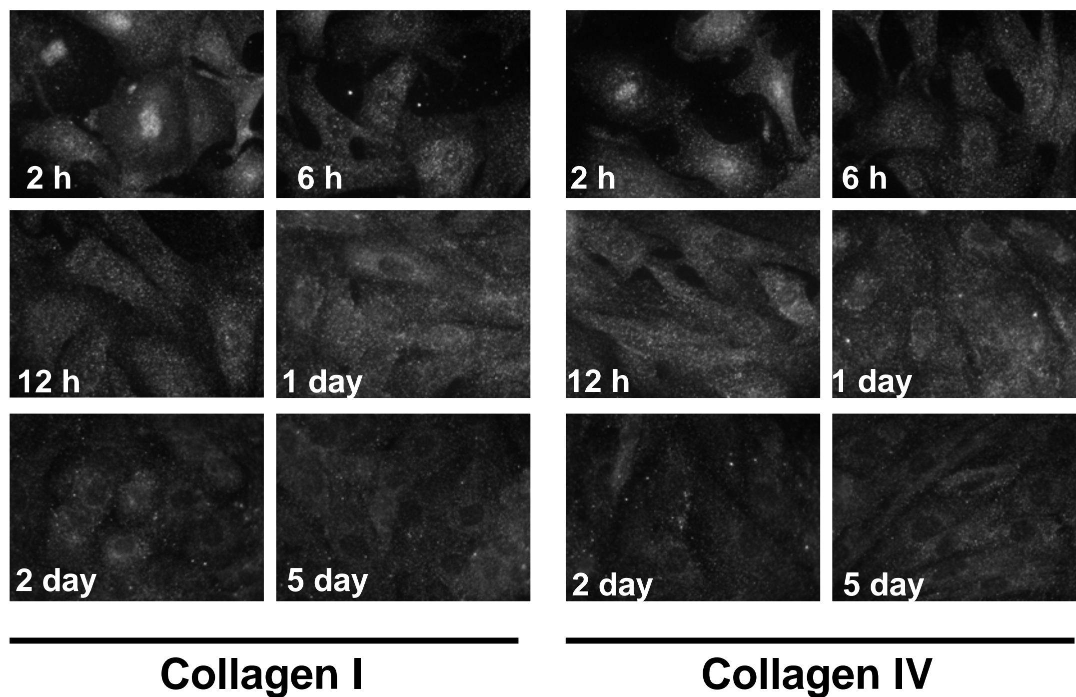
Figure S1 "Supplemental Material"





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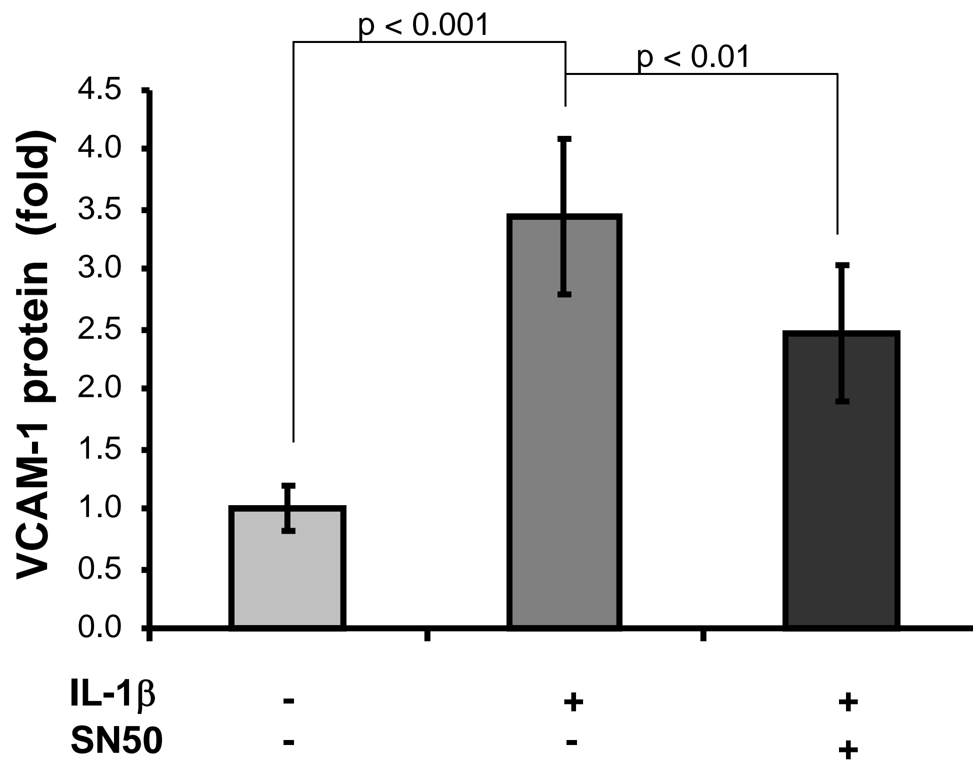

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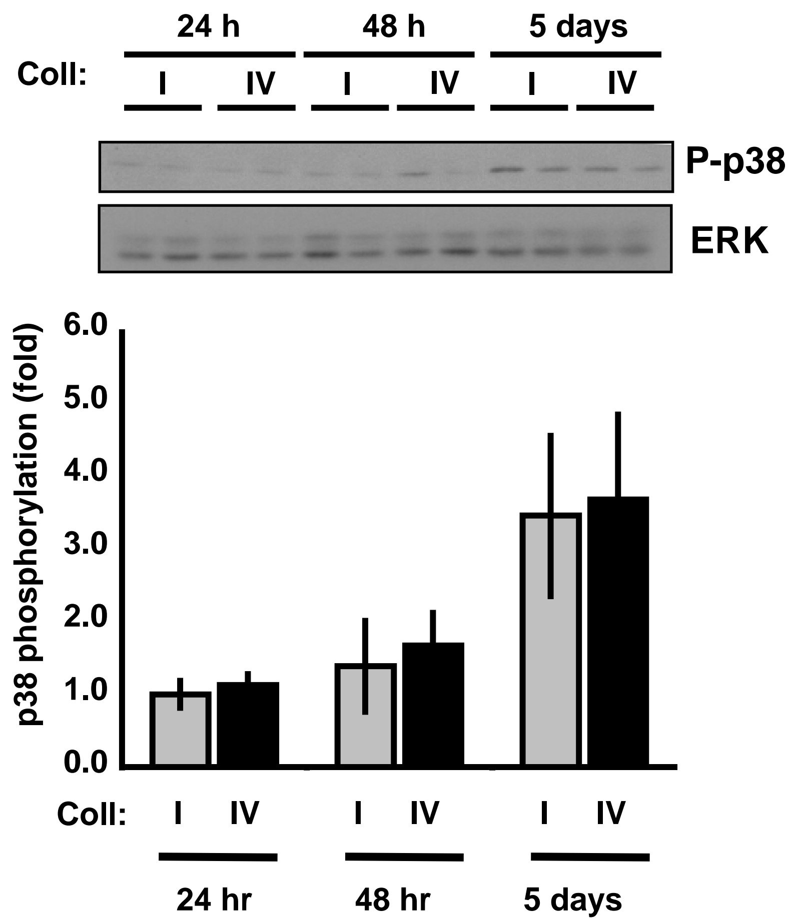
Figure S3 "Supplemental Material"



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