Supplement Material

Materials and Methods

Cell culture

Human coronary artery VSMC (Cascade Biologics, Portland, OR) (passages 3-6) were cultured in M199 media as described[1]. HUVEC and HUAEC (Lonza Walkerrsville Inc, MD, up to passage 8) were cultured on fibronectin in M199 media with 20% FBS, 50 μg/ml endothelial cell growth supplement (ECGS), 100 μg/ml porcine heparin (Sigma, St. Louis, MO) and penicillin-streptomycin. Cells were cultured in media with 2.5% (VSMC) or 0.5% FBS (HUVEC or HUAEC) for 16-24 hours prior to drug treatment. Untreated VSMC proliferate and do not spontaneously differentiate in media containing 2.5% FBS[1-3]. Vehicle was added for the maximum duration of the experimental treatment if not specified. Unless specified, human full-length recombinant adiponectin (HMW enriched) produced in HEK293 cells (RD172023100, BioVendor, Candler, NC) was utilized. Trimeric (RD172091100) and globular (RD172112100) recombinant adiponectin preparations were used where indicated. Rapamycin was purchased from LC Laboratories (Woburn, MA), AICAR from Toronto Research Chemicals Inc (Ontario, Canada) and Compound C from Calbiochem (San Diego, CA).

Cell lysis and western blotting

Cells were lysed as previously described[1]. Equal amounts of protein per lane were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and immunoblotted using primary and secondary antibodies as described[2], and primary antibodies against phospho-Thr 172 AMPK, AMPK-α (pan), AMPK-α1, AMPK-α2, phospho-ACC (Ser 79), total ACC, phospho-Ser 193 FoxO4 (Cell Signaling, Boston, MA) and total FoxO4 (Abcam, Cambridge, MA). Blots were developed using enhanced chemiluminescence reagents (Pierce, Rockford, IL) and quantitated using

ImageJ. Contractile proteins were normalized to GAPDH or β-tubulin. Phosphorylated proteins were normalized to corresponding total protein.

Transient transfection of siRNA and plasmid

Transient transfection of small interfering RNA (siRNA) in VSMC was performed via Nucleofector (Lonza Walkerrsville Inc, MD) as described[2]. For gene knockdown, 1 to 1.5 million cells were transfected with 2.5 to 3 μg siRNA and cultured in 2.5% FBS for 48 hours. Cells were treated with vehicle or adiponectin and harvested for Western blotting analysis as above. Akt1 (SMARTpool), Akt2 (4 different siGENOME duplexes) and nonsilencing siRNA (si*CONTROL*) were purchased from Dharmacon (Lafayette, CO), and AMPKα1, AMPKα2 and a matching nonsilencing control siRNA were purchased from Qiagen (Valencia, CA).

Transfection of HA-S6K1 or HA-Akt1 plasmids were as published[2,3]. Wild-type Flag-tagged FoxO4 plasmid was purchased from Addgene (Cambridge, MA). For gene overexpression, 1 million human VSMC were transfected with 10ug FoxO4 plasmid via Nucleofector and cultured in 10% FBS for 24 hours. VSMC were then cultured in 2.5% FBS and subjected to vehicle or adiponectin treatment for another 24 hours before harvesting for Western analysis. Transfection efficiencies routinely range from 40-70% based on a GFP control vector.

Adenoviral expression of rapamycin-resistant mutant S6K1

Infection of human VSMC with adenovirus encoding an HA-tagged, rapamycin-resistant, constitutively active mutant of S6K1 (ED3E) and green fluorescent protein (GFP), or with a control adenovirus (GFP), was performed as described[3]. Cells were infected with virus overnight and then washed, and media was changed to M199 with 2.5%FBS prior to adiponectin treatment.

Organ culture model

Pig femoral arteries were harvested and cut into 2 mm pieces (day 1), and maintained in M199 with 10% FBS. Drug or adiponectin was added on day 3. Media and rapamycin were refreshed every other day while fresh adiponectin was added daily. Tissue was homogenized on day 7 using our cell lysis buffer[1] for western blotting analysis.

Immunofluorescence

Human VSMC were cultured on glass coverslips and subjected to adiponectin or rapamycin treatment for the indicated time points, then washed with PBS and fixed in 1:1 methanol/acetone, blocked with 3% BSA in TBS-T, and incubated with anti-phospho-Ser 193 FoxO4 primary antibody in TBS-T with 1% BSA at 4ºC overnight. Cells were then incubated with AlexaFluor568-conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR) in TBS-T with 1% BSA for one hour, and DAPI staining was performed before mounting slides. Slides were analyzed using spinning disk confocal microscopy (Perkin-Elmer, Waltham, MA).

Statistical analysis

Densitometry results from triplicates or more were analyzed by GraphPad Prism software, and presented as the mean± standard error of the mean (SEM) unless otherwise specified. Significance of differences among groups was tested using one-way ANOVA with post-hoc Newman-Keuls test for multiple comparisons or paired t-test where indicated. A p value less than 0.05 was considered significant.

Supplemental Figure Legends

Supplemental figure I: Adiponectin induces VSMC differentiation marker expression and modulates the mTORC1 signaling pathway. A, VSMC were treated with HMW-enriched adiponectin (Adpn) at the indicated concentrations for 24h and subjected to western blotting analysis. B, Bar graphs represent densitometric quantification (mean fold induction plus standard error mean) of at least 3 separate replicates of Fig. 1B (main text). The number of replicates is indicated at the base of each bar. p-values (Newman-Keuls multiple comparison post hoc tests) are indicated above the bars: *$p<0.05$; **$p<0.01$, ***$p<0.001$ vs. vehicle.

Supplemental figure II: Adiponectin-induced VSMC differentiation requires AMPK. VSMC were pretreated with compound C (20 μM) for 30 min, followed by treatment with 5 μg/ml adiponectin for 24h and western blotting with the indicated antibodies. Blots shown are from a replicate experiment of Fig. 2A.

Supplemental figure III: A, hCASMC were starved in 2.5% FBS for 24 hrs, followed by treatment with vehicle or 5 μg/ml adiponectin for 8 hrs. The cells were then harvested and subjected to immunoprecipitation with specific Akt1 or Akt2 isoform antibodies. Each Akt isoform bound on the sepharose beads was eluted in loading buffer and analyzed by western using pan p- Akt (S473) antibody. Bar graphs represent densitometric quantification (mean fold induction plus standard deviation) of 2 separate experiments. B, VSMC were transfected with either pcDNA vector control or pcDNA-wt Akt1 plasmids and incubated for 48 hrs. Cells were then harvested and subject to western analysis with primary antibodies to contractile proteins and GAPDH. Bar graphs represent densitometric quantification (mean fold induction plus standard error of the mean) of SM-MHC or h-caldesmon normalized to GAPDH from 3 separate experiments. C, VSMC were transfected, treated, and analyzed as in Fig. 4. Bar graphs represent densitometric quantification (mean fold induction plus standard error mean) of 9 and 5 separate replicates of Fig. 4, respectively. *$p<0.05$ and ***$p<0.001$ vs. siControl.

Supplemental figure IV: Adiponectin induces FoxO4 phosphorylation and nuclear exclusion. VSMC were treated with 5 μg/ml adiponectin for the indicated time points, and subjected to immunohistochemistry staining using anti-phospho-Ser 193 FoxO4 primary and AlexaFluor568-conjugated secondary antibodies. Nuclei are stained with DAPI. Photographs from spinning disk confocal immunofluorescence microscopy are shown.

Supplemental figure V: Adiponectin and rapamycin have similar effects on VSMC phenotype. A, VSMC treated with 20nM rapamycin or 5 μg/ml adiponectin for the indicated time points were harvested and immunoblotted as in Fig. 1. B, VSMC were treated with 20nM rapamycin for the indicated time points, or with different concentrations of rapamycin for 24h prior to western blotting analysis as above. C, VSMC were treated with 50 nM rapamycin for the indicated time points and subjected to immunohistochemical staining as in Supplemental Figure IV. Photographs from spinning disk confocal immunofluorescence microscopy are shown.

Supplemental figure VI: Model of adiponectin and rapamycin signaling in VSMC and EC. A, Model of adiponectin and rapamycin-induced VSMC differentiation. Adiponectin activates AMPKα2, which in turn inhibits the mTORC1/S6K1 pathway, relieving S6K1-dependent feedback inhibition of IRS-1. The resulting stabilization of IRS-1 activates PI3K and Akt2 signaling, which promotes VSMC differentiation via inhibition of FoxO4 transcription factor. Rapamycin induces a similar signaling cascade through direct inhibition of mTORC1. B, Model of adiponectin and rapamycin signaling in EC. Rapamycin significantly while adiponectin only slightly inhibits mTORC1. Also, chronic rapamycin treatment inhibits while adiponectin may preserve mTORC2 assembly, a PDK2 which phosphorylates Akt at Ser 473, therefore rapamycin significantly inhibits while adiponectin preserves Akt phosphorylation at Ser 473 in EC. Both mTORC1 and Akt are very important in promoting EC growth and survival.

References

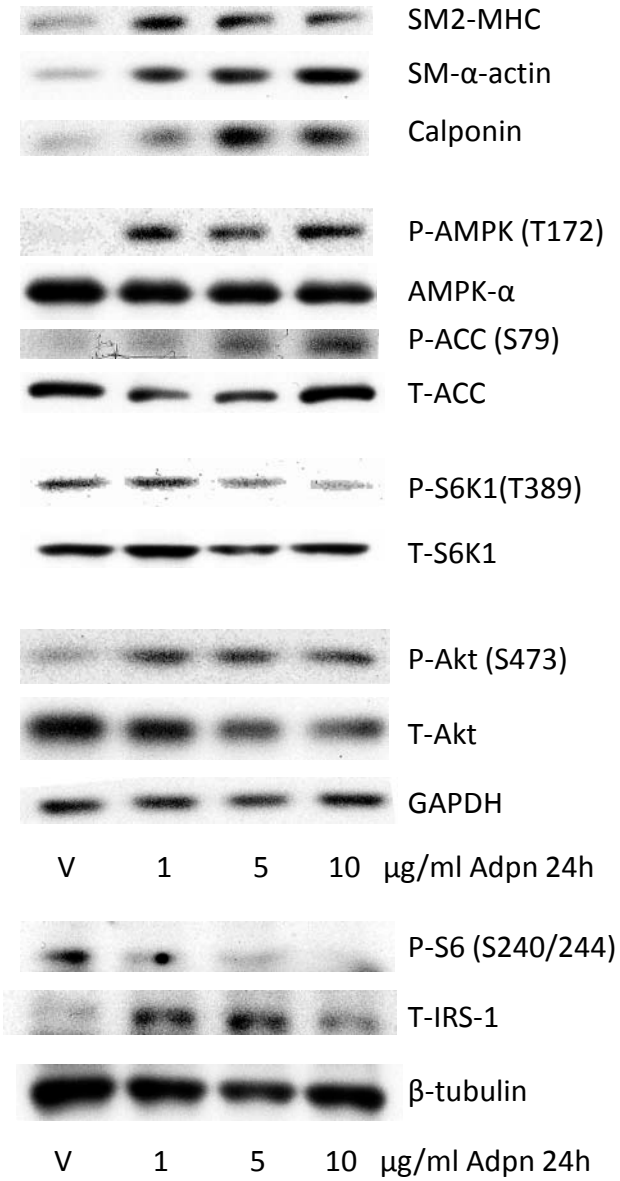
1. Fetalvero KM, Shyu M, Nomikos AP, Chiu YF, Wagner RJ, Powell RJ, Hwa J, Martin KA. The prostacyclin receptor induces human vascular smooth muscle cell differentiation via the protein kinase A pathway. *Am J Physiol Heart Circ Physiol* 2006;290:H1337-H1346.

2. Martin KA, Merenick BL, Ding M, Fetalvero KM, Rzucidlo EM, Kozul CD, Brown DJ, Chiu HY, Shyu M, Drapeau BL, Wagner RJ, Powell RJ. Rapamycin promotes vascular smooth muscle cell differentiation through insulin receptor substrate-1/phosphatidylinositol 3-kinase/Akt2 feedback signaling. *J Biol Chem* 2007;282:36112-36120.

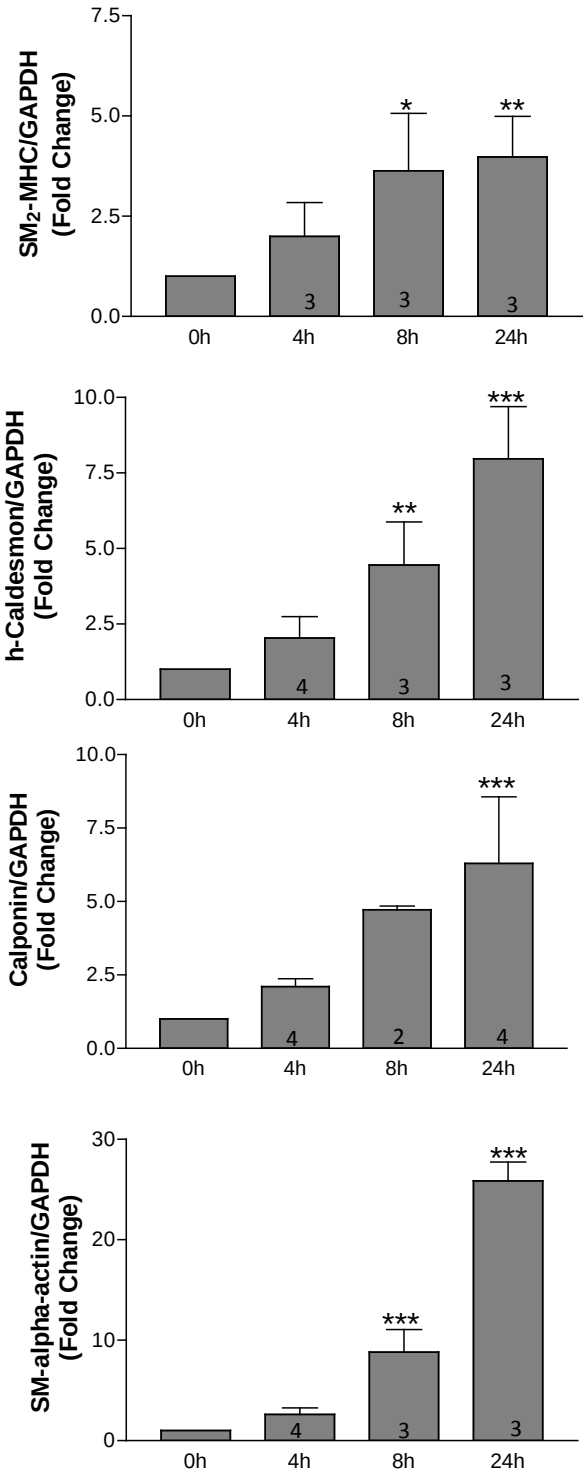
3. Martin KA, Rzucidlo EM, Merenick BL, Fingar DC, Brown DJ, Wagner RJ, Powell RJ. The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation. *Am J Physiol Cell Physiol* 2004;286:C507-C517.

Supplemental Fig. I Adiponectin Induces VSMC Differentiation

A. Dose-Effects

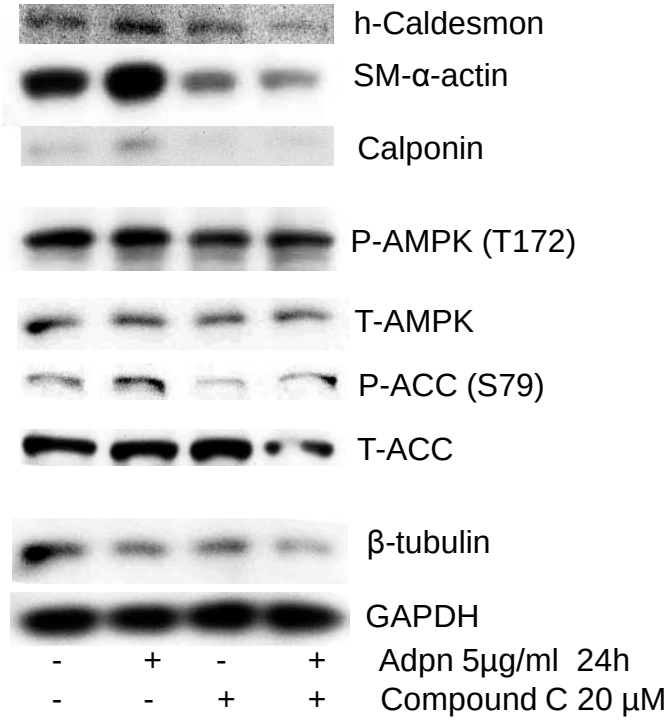


B. Quantitations of the Time-Course

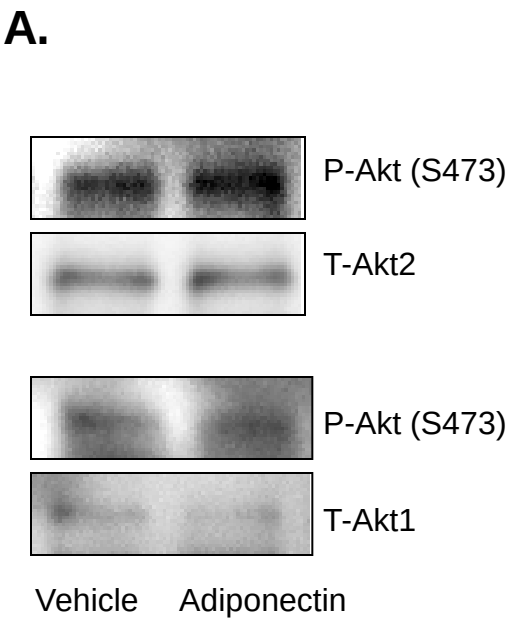


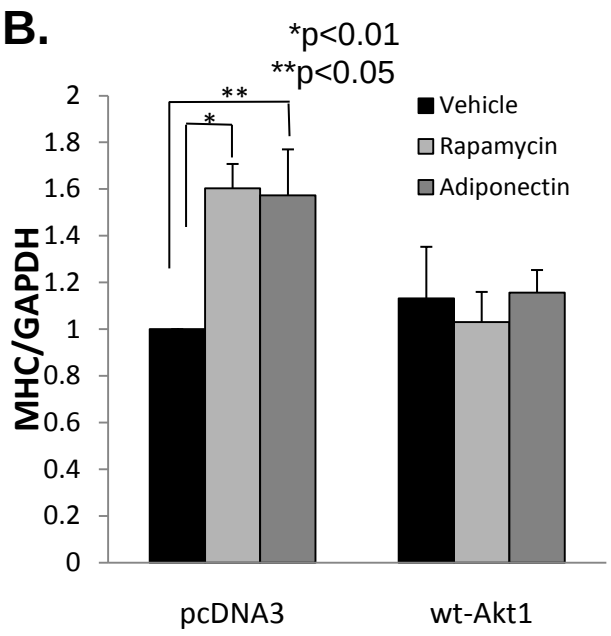
*p<0.05, **p<0.01,***p<0.001

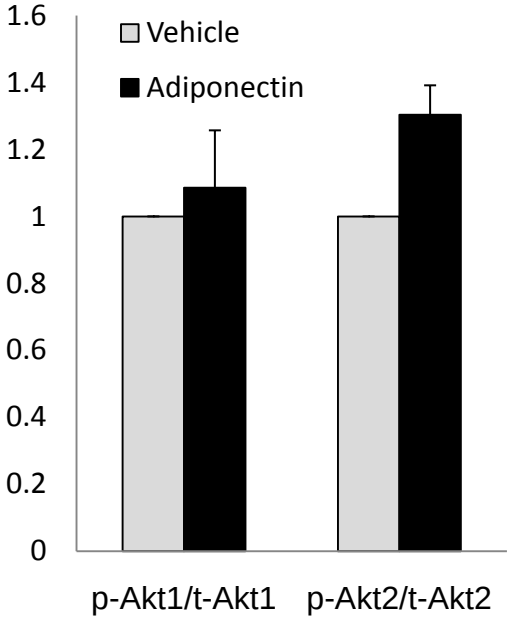
Supplemental Fig. II Adiponectin-Induced VSMC Differentiation Is Mediated by AMPK Activation

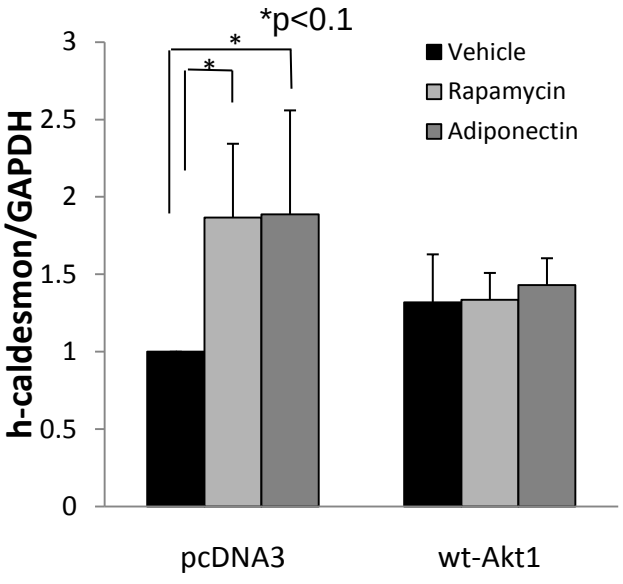


Supplemental Fig. III Akt isoform effects



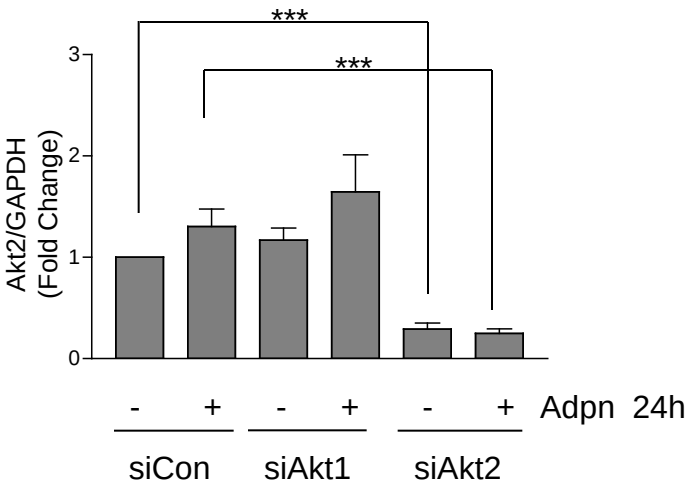




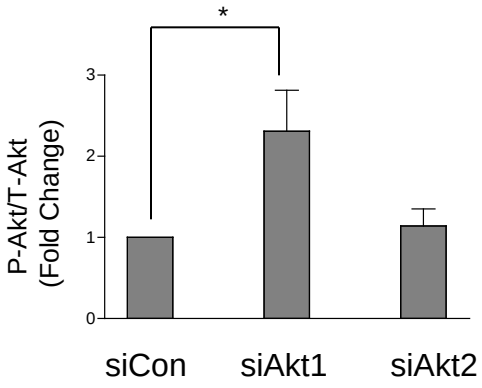


Supplemental Fig. III Akt Isoform Effects

C.

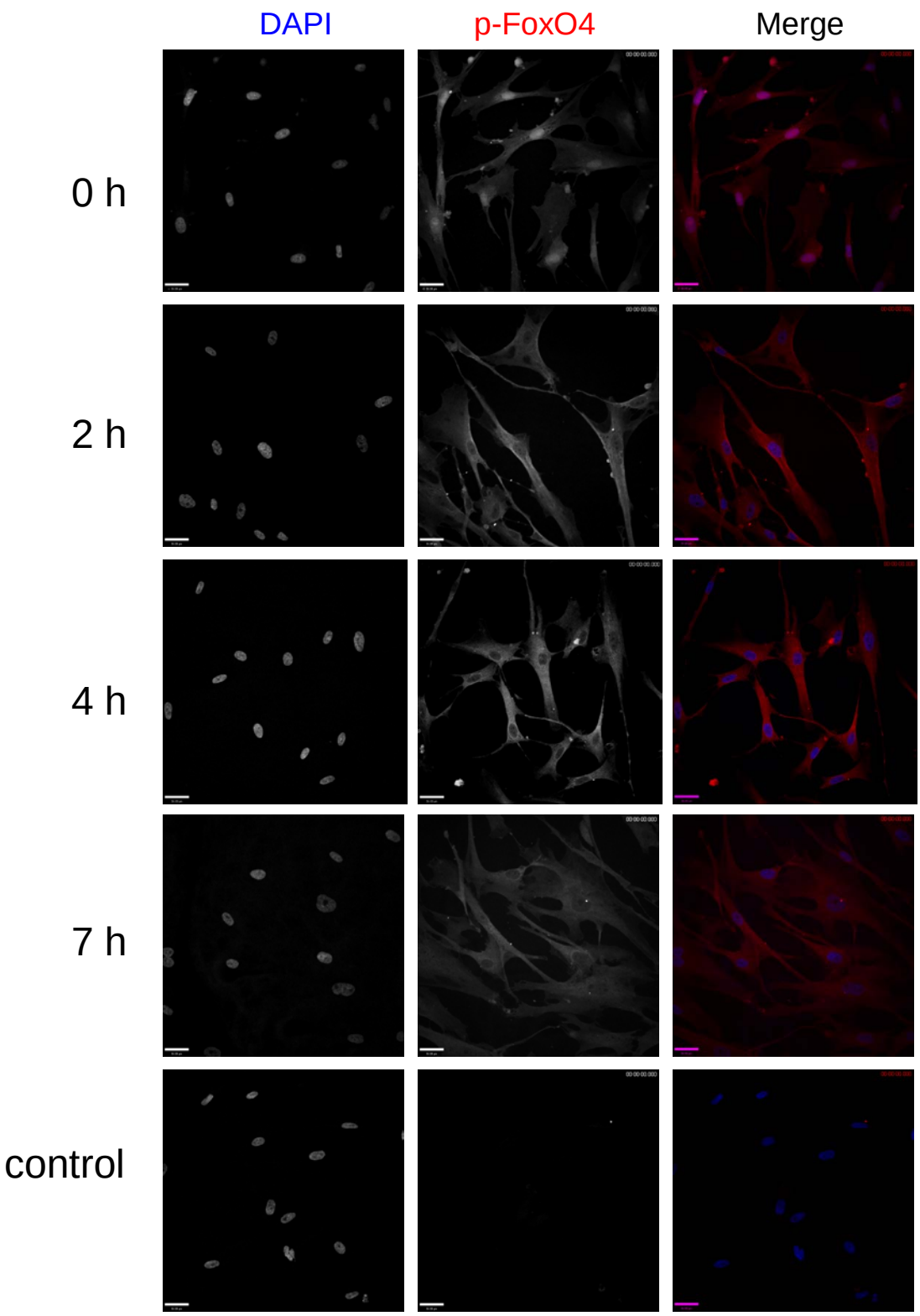


N=9, ***p<0.001

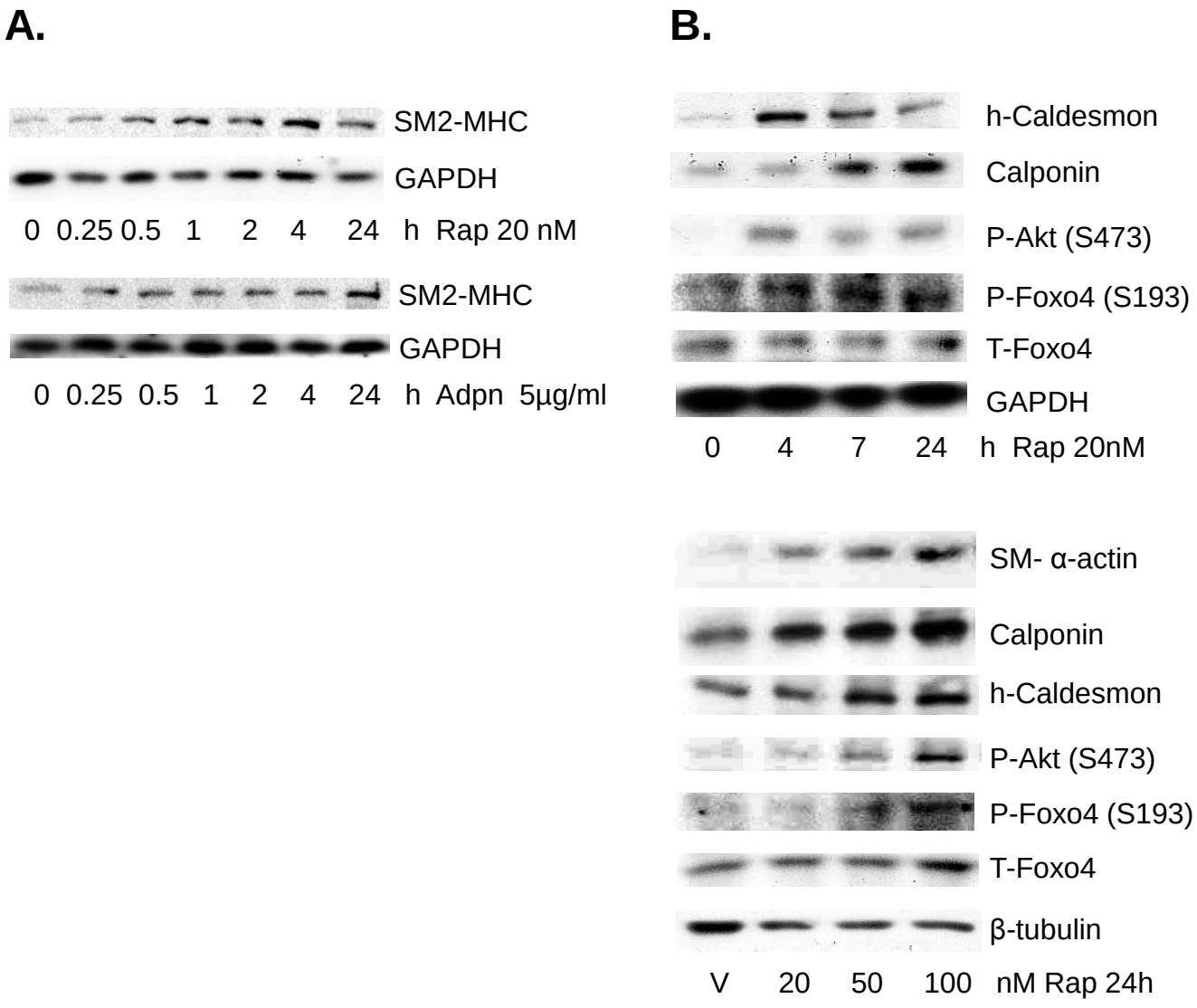


N=5, *p<0.05

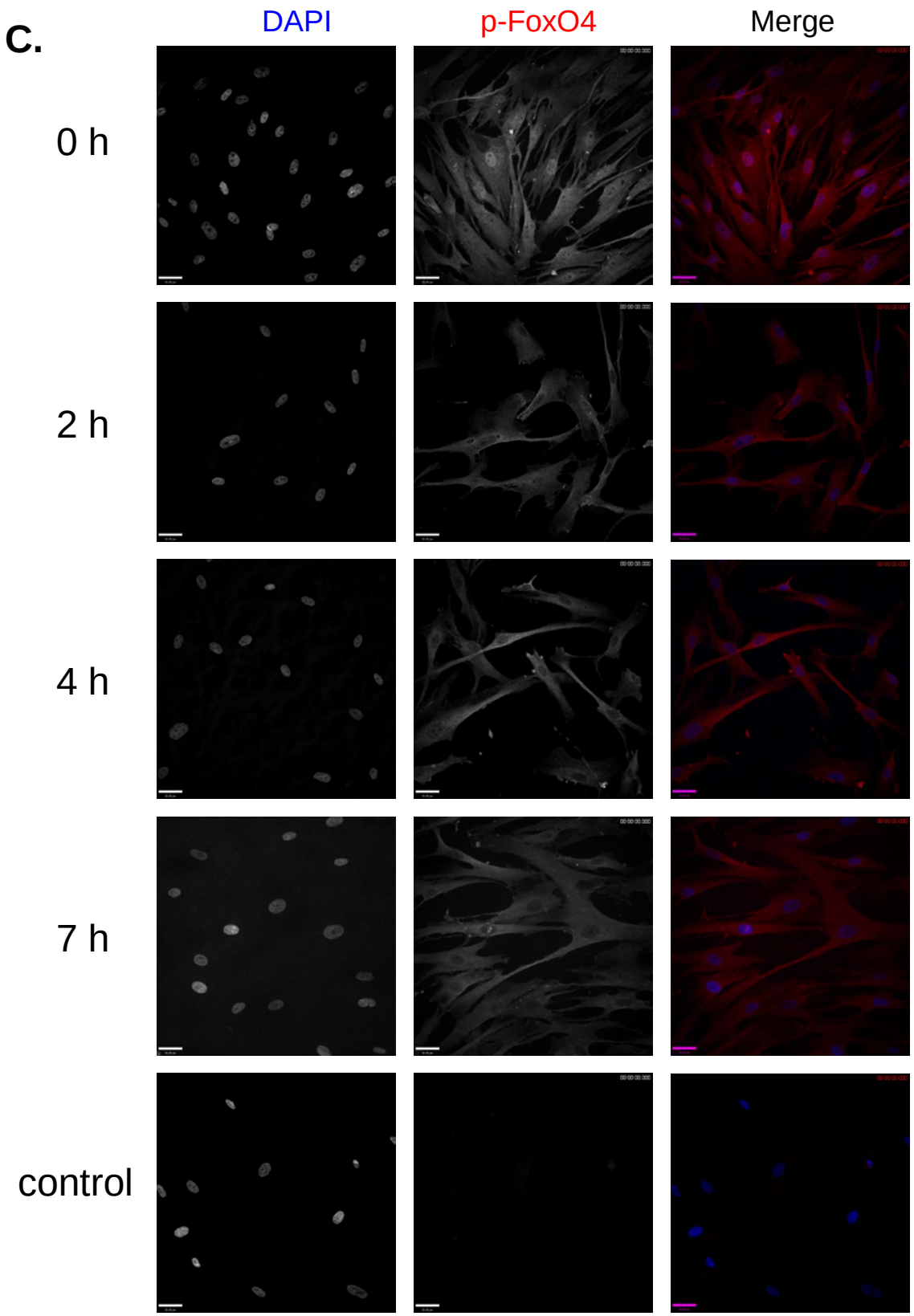
Supplemental Fig. IV Adiponectin regulates FoxO4 phosphorylation and nuclear exclusion



Supplemental Fig. V Adiponectin and Rapamycin Have Similar Beneficial Effects on VSMC Phenotype

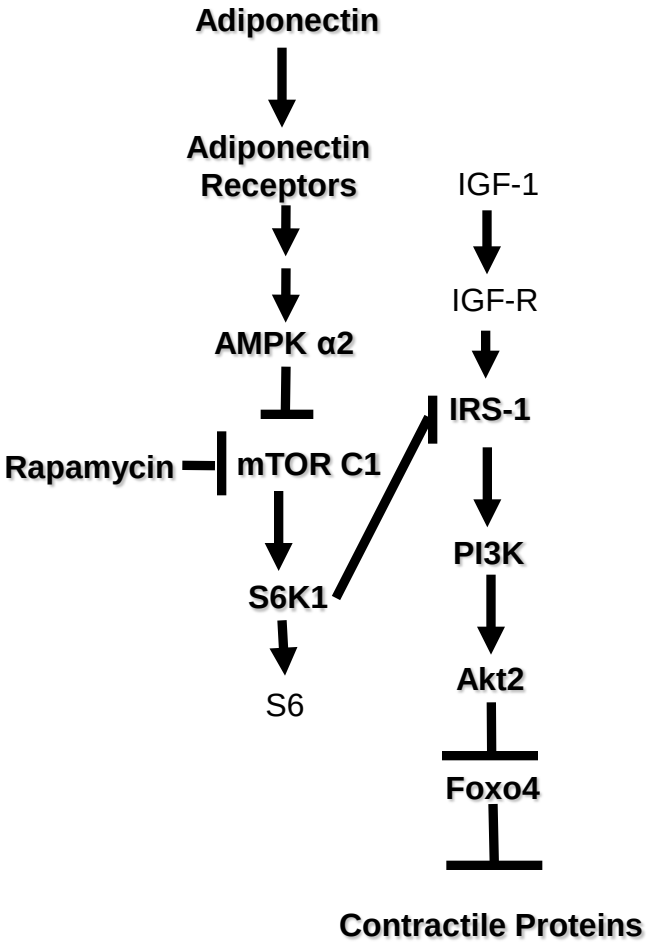


Supplemental Fig. V Rapamycin regulates FoxO4 phosphorylation and nuclear exclusion



Supplemental Fig. VI Model of Adiponectin and Rapamycin Signaling in VSMC and EC

A. VSMC



Supplemental Fig. VI Model of Adiponectin and Rapamycin Signaling in VSMC and EC

B. EC