SUPPLEMENTAL MATERIAL

Detailed Methods

Regents

Forskolin, PGE_1, isobutylmethylxanthine (IBMX) were purchased from Sigma (St. Louis, MO). Indomethacin SB203580, U73122, LY294002, wortmannin, H89, bisindolylmaleimide I (BIS) and phorbol-12-myristate-13-acetate (PMA) were from Calbiochem (San Diego, CA); D-IP3, L-IP3 and neomycin sulfate were from Alomone labs (Jerusalem, Israel); 8-p-methoxyphenylthon-2'-O-methyl-cAMP (Me-cAMP) and N6-Benzoyladenosine-cAMP (Bnz-cAMP) were from Biolog Life Science Institute (Bremen, Germany).

Antibodies

Anti-AC2, AC5/6 and GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); Anti-phospho-p38, p38, phospho-MKK3/6 and MKK3 antibodies were from Cell Signaling Technology (Danvers, MA); Anti-VASP and FITC-conjugated CD31 antibodies were from Abcam (Cambridge Science Park, Cambridge); Anti-phospho-VASP (Ser236), APC/Cy7-conjugated CD45 and caveolin 1 antibodies were from Calbiochem (San Diego, CA), Biolegend (San Diego, CA) and BD Biosciences (Franklin Lakes, NJ), respectively.

Organ Culture

DA on the 19th day of gestation were infected for 2 h with 1.2 x 10^7 plaque-forming unit/ml of the AC2, AC6, AC2+AC6 or LacZ adenovirus in 0.5% fetal calf serum containing Dulbecco's modified Eagle's medium in humidified 5% CO2 and 95% ambient mixed air at 37 °C. The segments were cultured for 2 days and fixed in 10% buffered formalin. Paraffin embedded sections were subjected to Elastica van Gieson staining or staining for hyaluronan-binding protein as described previously. Morphometric analyses were performed using Image J software. Intimal cushion formation was defined as ((neointimal area)/(medial area)) x 100%.

DA on the 19th day of gestation were incubated with FD1 (10^{-5}mol/L) or FD6 ($10^{-5.5}$mol/L) in 0.5% fetal calf serum containing Dulbecco's modified Eagle's medium in humidified 5% CO2 and 95% ambient mixed air at 37 °C. The segments were cultured for 3 days and fixed and analyzed as same as above.

cAMP Production by Radioimmunoassay

DASMCs grown on 24-well plates were serum-starved for 24 h and assayed for cAMP production by 20-min incubation with 0.2 mmol/L IBMX, followed by the addition of each drug for an additional 10 min. Reactions were terminated by aspiration of the media and the addition of 400 μl of ice-cold trichloroacetic acid (7.5%) to each well. Samples were acetylated using 10

μL of acetic anhydride and 20 μl of triethylamine (Sigma, St Louis, MO), and 50 μL aliquots were incubated with 12,000 cpm of 125I-cAMP (Perkin Elmer, Waltham, MA) and 50 μL of rabbit anti-cAMP antibody (diluted 1:3000, Millipore, Billerica, MA) overnight at 4°C. Fifty microliters of goat anti-rabbit antibody with magnetic beads (QIAGEN, Tokyo, Japan) was added and incubated with each mixture for 1 h with constant shaking at room temperature. Separation of bound from free antibodies was achieved by filtration (Millipore, Billerica, MA), and bound radioactivity was counted and compared with a standard curve. Production of cAMP was normalized to the amount of protein per sample.

Measurement of Isometric Tension of the Vascular Rings of DA

After the timed-pregnant rats were anesthetized with an overdose of pentobarbital (100mg/kg), the fetuses at e21 were delivered by cesarean section. The vascular ring of DA was placed in a tissue bath and kept at 37°C. Two tungsten wires (30 μm in diameter) were threaded into the lumen and the preparation was mounted in a 2-channel myograph (Dual Wire Myograph System-410A, Unique Medical, Tokyo, Japan). After stabilization, the vascular ring was exposed to 10^{-6}M of indomethacin-contaning Krebs-Henseleit solution at least for 10min at 37°C. After a plateau contraction had been attained with indomethacin, FD1 or FD6 was added to stimulate vasodilatation. After the force reached a new steady state, the concentration of FD1 or FD6 was increased from 10^{-8} to 10^{-5} M. At the end of all experiments, ductal contraction was induced by potassium-enriched solutions (NaCl, 22mM; KCl, 120mM; CaCl2, 1.5mM; glucose, 6mM; MgCl, 1mM; HEPES, 5mM; pH7.4) to confirm the viability.

Flow cytometric analysis and cell sorting

Dissociated single cells from rat e21 DA and aorta were analyzed with a MoFlo cell sorter (Dako Cytomation, Fort Collins, CO) [2, 3]. FITC-conjugated anti-CD31 (1:10) and APC/Cy7-conjugated anti-CD45 (1:40) were added to cell suspensions at a concentration of 3 x 10^6 cells / ml and incubated at 4°C for 30 min. Cells were then washed with PBS containing 3% FBS before cell sorting. Dead cells were excluded by propidium iodide staining. Erythrocytes were not detected by anti-erythroid antibody. Cell populations of CD31+/CD45- and CD31-/CD45- were distinguished from each other. Different cell fractions were gated based on the intensity of FITC and APC/Cy7 and sorted cells. Sorted cells were collected by centrifugation at 1500 rpm for 5 min, and pellets were suspended in RNA lysis buffer and stored at –80° C until use. RNA was extracted using the RNeasy micro kit (QIAGEN).

PKA Activity assay

PKA activity was determined by an enzyme-linked immunosorbent assay using lysates of DASMCs serumstarved for 24 h and stimulated with drugs of interest. Cells were then scraped into lysis buffer (20 mM Mops, 50 mM β-glycerol phosphate, 50 mM NaF, 1 mM NaVO4, 5 mM EGTA, 2 mM EDTA, 1% Nonidet P-40, 1 mM DTT, and protease inhibitor

mixture from Sigma), incubated for 10 min, and then passed through a 25-gauge needle 10 times. Homogenates were centrifuged at 15,000 g for 15 min. PKA activity was measured in the resulting supernatant.

Rap1 pull-down assay.

Rap1 activity was measured using the EZ-Detect RAP1 activation kit (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions. Cells were serum-starved for 24 h, incubated with drugs for 15 min. Cell lysates were incubated with Rap-binding domain RalGDS-RBD fused to a glutathione S-transferase disk. After repeated washing steps, bound GTP-Rap1 was removed from the disk by boiling in a SDS sample buffer and analyzed by Western blotting using an anti-Rap1 antibody.

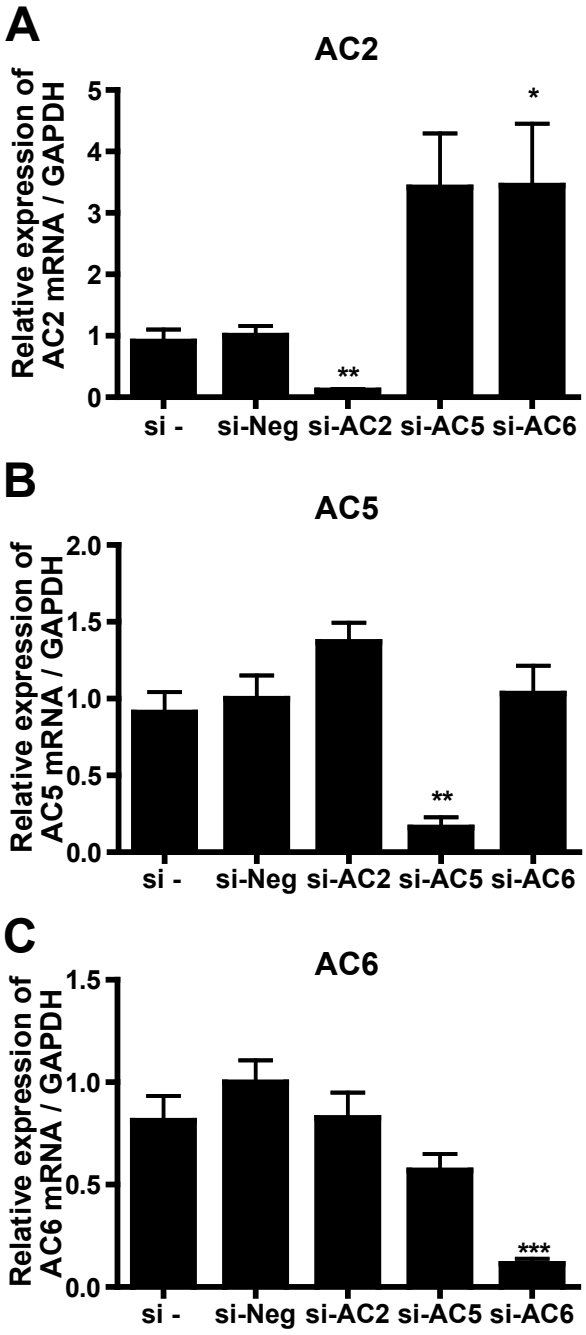
Protein Fractionation by the Sucrose Gradient Method

Caveolae fractions were separated by the sodium carbonate-based detergent-free method. DA SMCs were homogenized in a solution containing 0.5 M sodium carbonate (pH 11) and protease inhibitors with four 20-s bursts of a sonicator. The homogenates were adjusted to 45% sucrose by adding 90% sucrose in a buffer containing 25 mM Mes (pH 6.5) and 0.15 M NaCl and placed at the bottom of an ultracentrifugation tube. A 5-35% discontinuous sucrose gradient was formed above and centrifuged at 39,000 rpm at 4°C for 16 h in a Beckman SW-41Ti rotor. From the top of the tube, 13 fractions were collected. Caveolae fractions were accumulated in fractions 4-6. To analyze the intracellular localization of AC2 or AC5/6, an equal volume from each fraction was subjected to immunoblotting.

[^{3}H]Thymidine incorporation

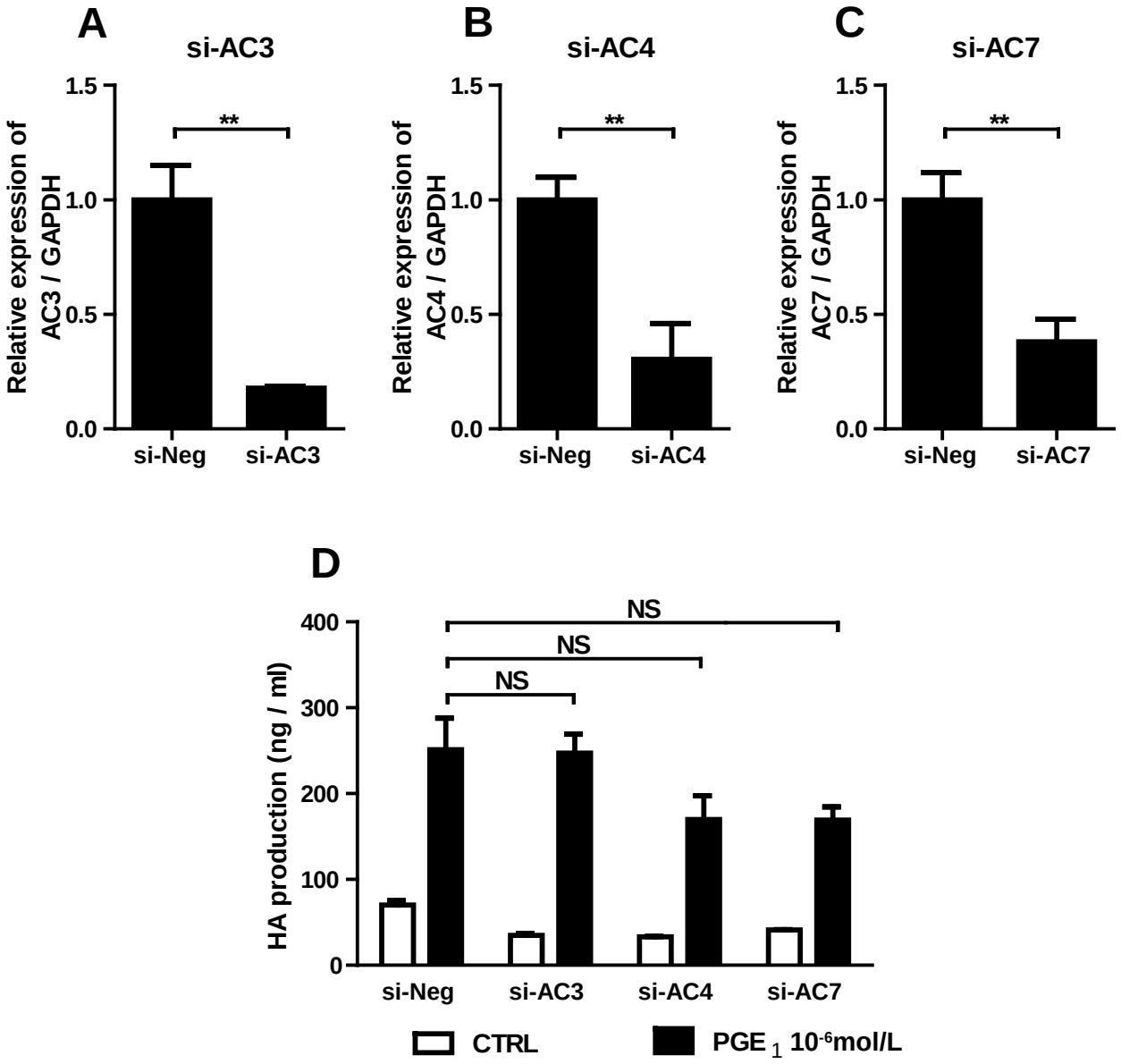
Cell proliferation was measured using [^{3}H]thymidine incorporation as described previously. Briefly, The SMCs were reseeded into a 24-well culture plate at an initial density of 1×10^5 cells per well. LacZ, AC2, AC6 or AC2+AC6 was overexpressed by the use of adenovirus (5MOI) for 48 h before addition of drug (FD1, FD6, forskolin, or PGE_1) and 1 μCi of [methyl ^{3}H]thymidine for 4 h at 37° C.

Online Figure I



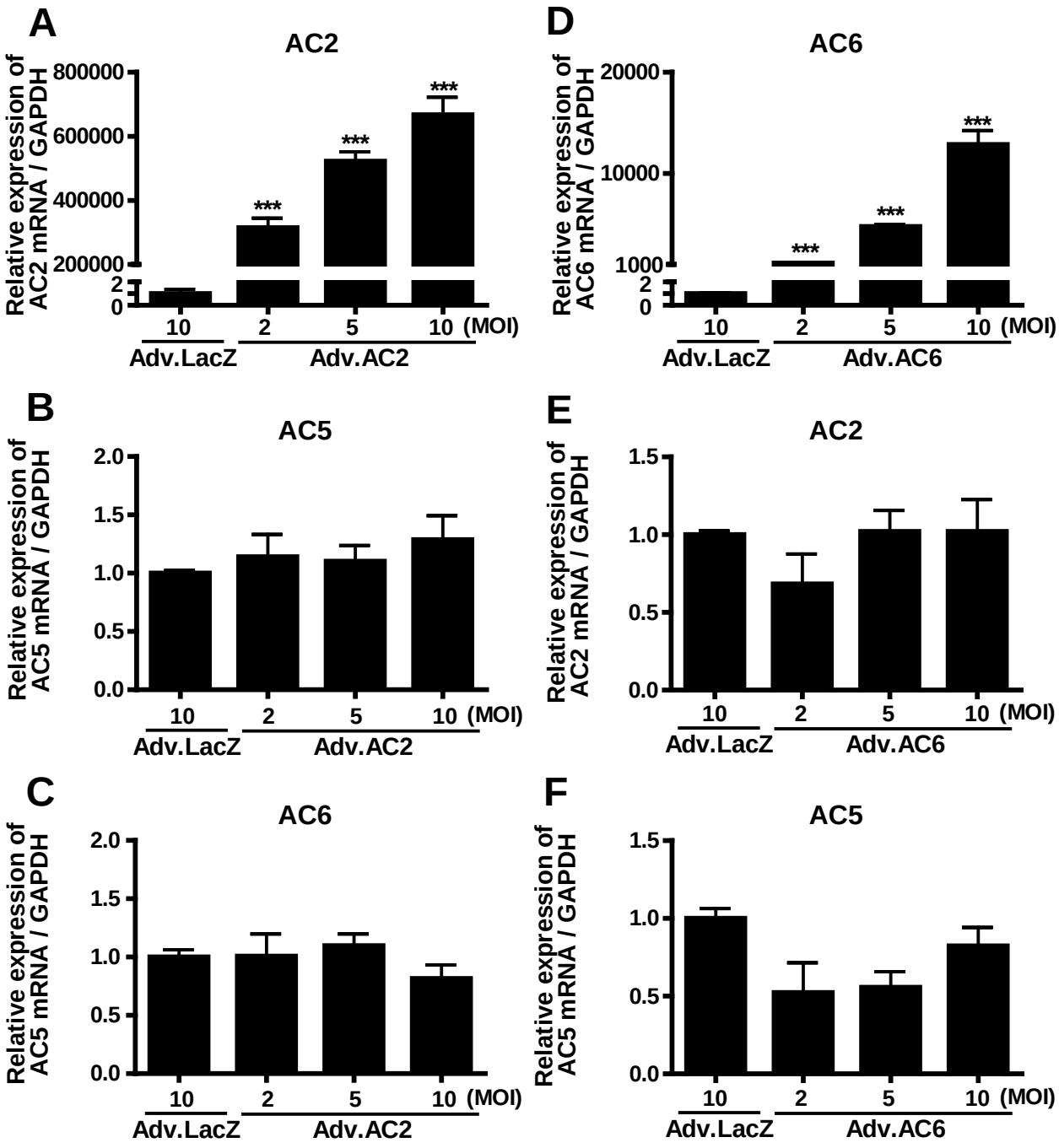
Online Figure I
(A) Expression of AC2 mRNA was silenced by AC2-targeted siRNA in DASMCs. AC2 mRNA expression was increased by AC6-targeted siRNA (n=6). (B) AC5 mRNA expression was significantly decreased by AC5-targeted siRNA, but not by AC2-and AC6-targeted siRNA (n=6). (C) AC6 mRNA was decreased by AC6-targeted siRNA, but not by AC2-and AC5-targeted siRNA (n=6). *P<0.05, **P<0.01 and ***P<0.001 compared to si-Neg. Data are from at least three independent experiments.

Online Figure II



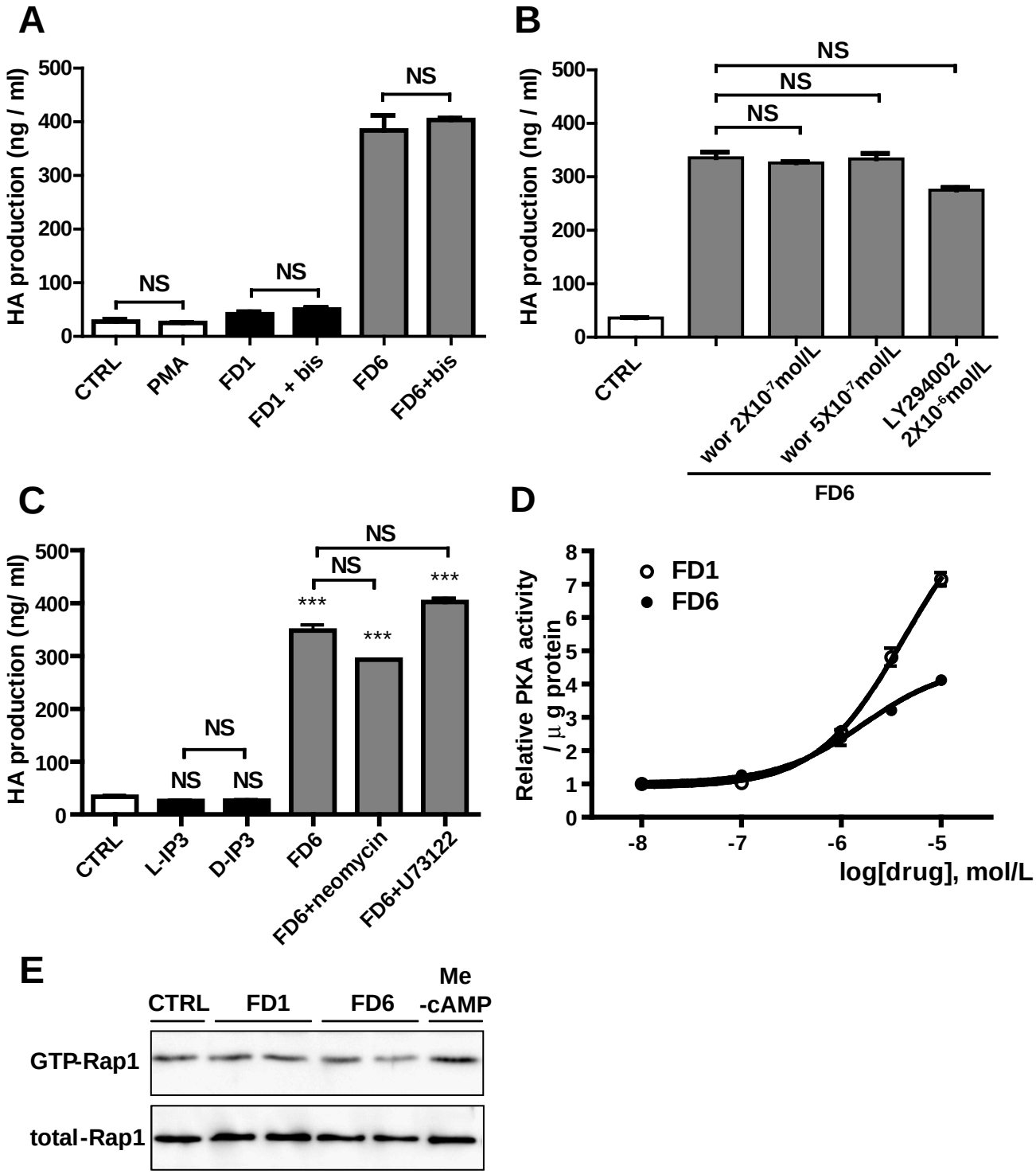
Online Figure II
(A-C) Expression of AC3, AC4 or AC7 mRNA was silenced by AC3-, AC4-or AC7-targeted siRNA in DASMCs. (n=4). (D) AC3-, AC4-or AC7-targeted siRNA did not attenuate PGE_1-induced hyaluronan (HA) production (n=6). **P<0.001. Data ware from three independent experiments.

Online Figure III



Online Figure III
(A, B and C) Effect of overexpression of AC2 on mRNA expression of AC2, AC5 and AC6. Overexpression of AC2 significantly increased AC2 mRNA, but not AC5 and AC6 (n=6). (D, E and F) Overexpression of AC6 significantly increased AC6 mRNA, but not AC2 and AC5 (n=6).
***P<0.001 compared to Adv.LacZ. Data are from at least three independent experiments.

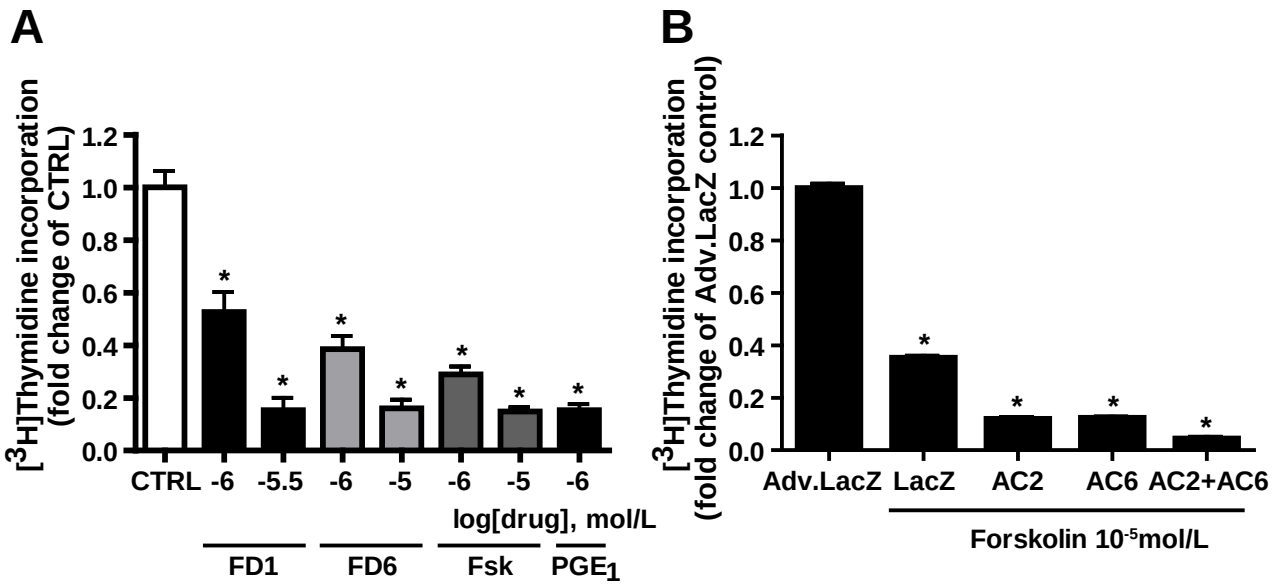
Online Figure IV



Online Figure IV

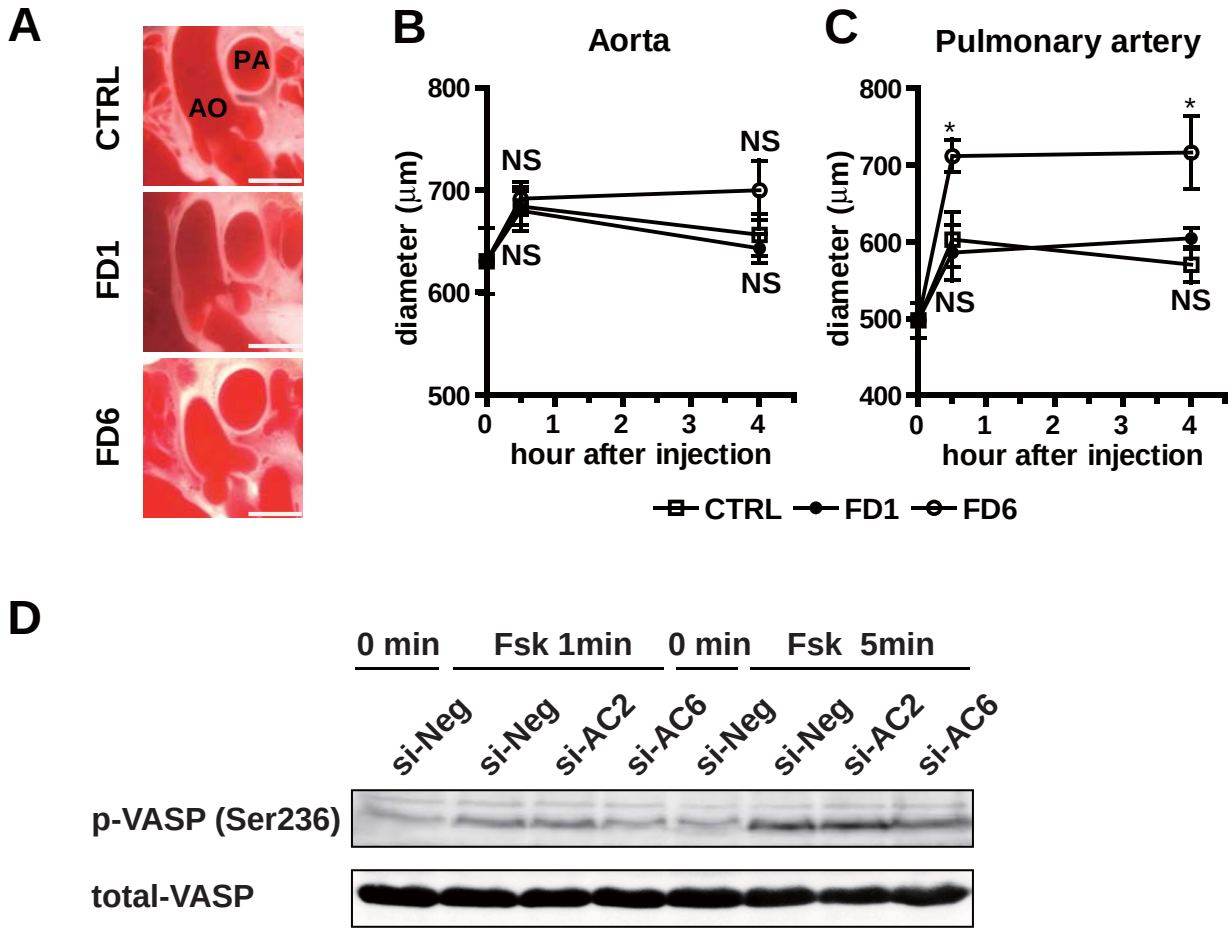
(A) PMA (phorbol-12-myristate-13-acetate, PKC activator, 10^{-6}mol/L) and bis (bisindolylmaleimide I, PKC inhibitor, 10^{-6}mol/L) did not affect hyaluronan (HA) production under basal or FD1/FD6 treatment. (n=6). (B) Wortmanin (wor, PI3K inhibitor) or LY294002 (PI3K inhibitor) did not affect FD6-induced hyaluronan production. (n=6) (C) IP3 receptor agonist (D-IP3, 10^{-5}mol/L) did not increase hyaluronan production compared to an inactive IP3 receptor agosnit (L-IP3, 10^{-5}mol/L). Neomycin sulfate (PLC inhibitor,10^{-5}mol/L) and U73122 (PLC inhibitor, 10^{-5}mol/L) did not affect FD6-induced hyaluronan production. (n=6) (D) FD1 and FD6 increased PKA activity in a dose -dependent manner. PKA was activated in accordance with cAMP produced by FD1 or FD6, respectively (n=8-10). (E) There was not different in GTP-Rap1 protein expression between DASMCs treated with FD1 ($10^{-5.5}$mol/L) and FD6 (10^{-5}mol/L). Me-cAMP: 8-p-methoxyphenylthon-2'-O-methyl-cAMP, $5X10^{-5}$mol/L. ***P<0.001. Data are from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 compared to si-Neg.

Online Figure V



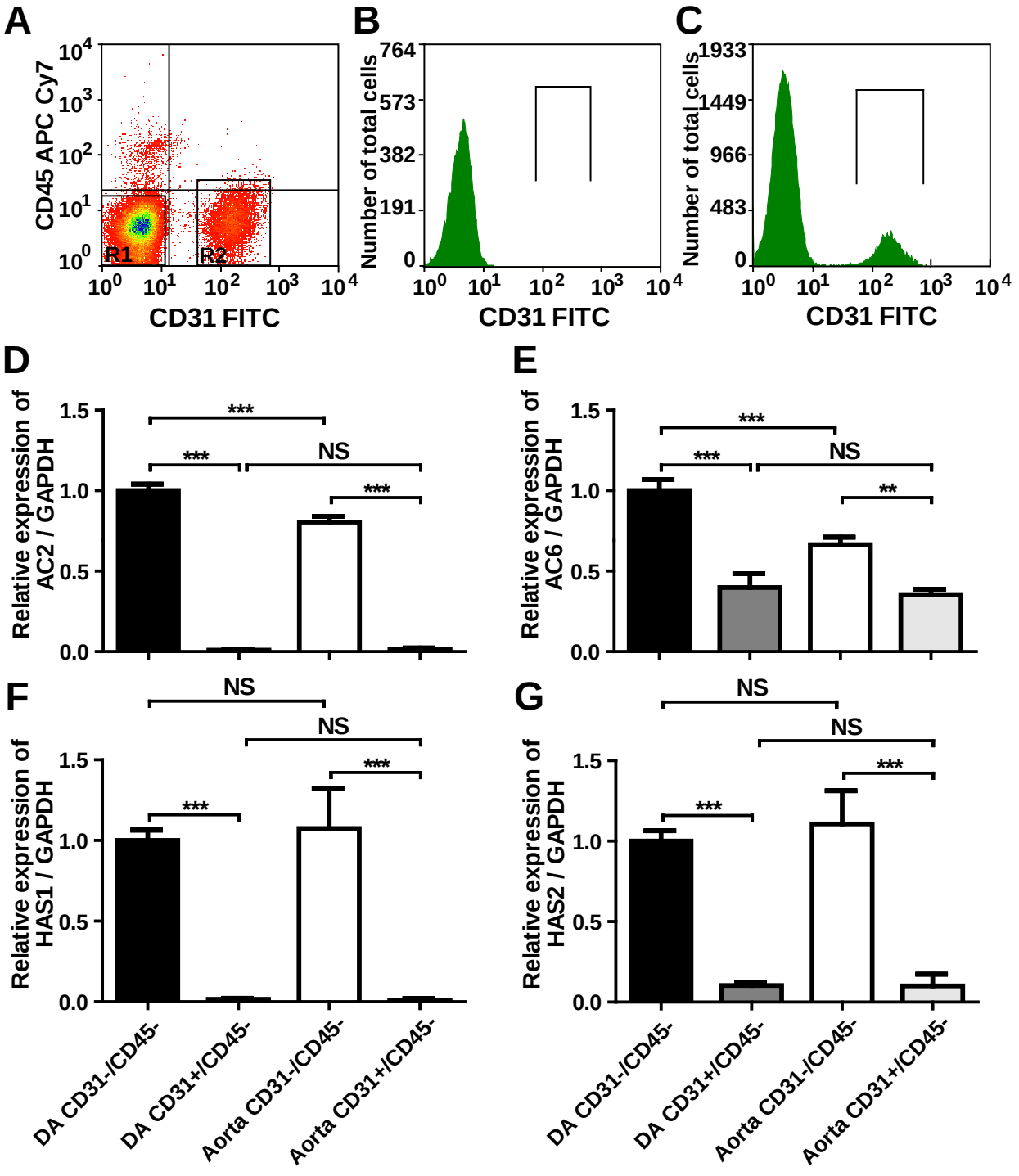
Online Figure V
(A) DNA synthesis was measured in DASMCs treated with FD1, FD6, forskolin (Fsk) or PGE_1 for 4 h. (n=6) (B) Forskolin-indueced supression of DNA synthesis was enhanced in DASMCs transfected with Adv.AC2 or Adv.AC6 (n=6). *P<0.01 compared to CTRL or Adv.LacZ control. Data are from three independent experiments.

Online Figure VI



Online Figure VI
(A) Representative images of the rat aorta and pulmonary arteries treated with FD1 or FD6. (B and C) The diameter of rat ascending aorta was not changed by FD1 or FD6, whereas that of main portion of pulmonary arteries were increased by FD6, but not by FD1 (n=5-7). *P<0.05 compared to before injection. Scale bars, 1 mm. (D) Representative images of phophorylation of VASP (Vasodilator -stimulated phosphoprotein) induced by forskolin (Fsk,10^{-5}mol/L) in DASMCs treated with si-Neg, si-AC2 or si-AC6. Data are from three independent experiments.

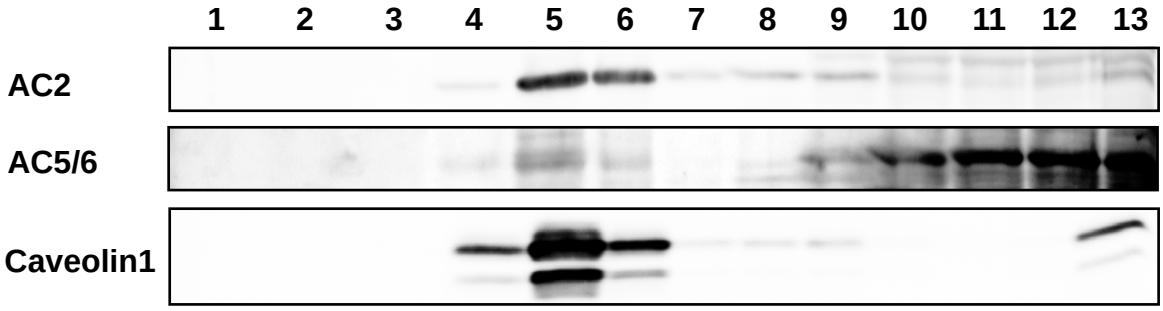
Online Figure VII



Online Figure VII

(A) Flow cytometric analysis using FITC-conjugated anti-CD31 and APC/Cy7-conjugated anti-CD45 antibodies in the rat e21 DA tissue. The gates R1 and R2 represent CD31-/CD45- SMCs and CD31+/CD45- endothelial cells, respectively. (B and C) Total number of CD31+ cells in the rat e21 DA tissue. Negative staining without a primary antibody was shown in B. (D and E) AC2 and AC6 mRNA expression in SMCs or endotherial cells of the rat e21 DA or aorta tissue (n=4). (F and G) HAS1 and HAS2 mRNA expression in SMCs or endotherial cells of the rat e21 DA or aorta tissue (n=4). **P<0.01 and ***P<0.001.

Online Figure VIII



Online Figure VIII
Representative images of AC2, AC5/6 and caveolin1 protein expression separated by a sucrose gradient method. Fraction number 4-5 and 9-13 indicates buoyant and heavy fractions, respectively. Data are from three independent experiments.

Online Table I Oligonucleotides used for RT-PCR

Gene	Accession number	Forward (5'-3')	Reverse (5'-3')	Size (bp)
AC1	XM_223616	accagccaagaggatgaagtt	acaccagcagcagcaggacag	446
AC2	NM_031007	TaqMan(R) Gene Expression Assays, Rn00578713_m1		-
AC3	NM_130779	accgtaagcaccgaaagg	caacatctcgtcagccaca	134
AC4	NM_019285	ggtcatctcatccctctcaca	caatgctcgctccatcag	163
AC5	NM_022600	tgtccttggcctcagaaagt	tccccgttcaggtagttgag	132
AC6	L01115	caaaggaagggacgccgagagg	tggggacagatcacgggactagga	419
AC7	XM_226333	gctgctgctgaagcccaagtt	aatcactccagcaatcacagg	256
AC8	NM_017142	ttcacttgagcctagcctcg	ggatgtagatgcggtggaac	627
AC9	NM_001106980	gacggtcttgtgtggcatc	ttagctgtctctctttcaactggtc	227
HAS1	NM_172323	TaqMan(R) Gene Expression Assays, Rn00597231_m1		-
HAS2	NM_013153	TaqMan(R) Gene Expression Assays, Rn00565774_m1		-

Online Table II Oligonucleotides used for siRNA

Gene	siRNA
AC2	HP GenomeWide siRNA SI00269507 (QIAGEN)
AC3	Stealth RNAi™ siRNA RSS 329955 (Invitrogen)
AC4	Stealth RNAi™ siRNA RSS 328959 (Invitrogen)
AC5	HP GenomeWide siRNA SI01485715 (QIAGEN)
AC6	HP GenomeWide siRNA SI00253246 (QIAGEN)
AC7	Stealth RNAi™ siRNA RSS 373110 (Invitrogen)
control	All stars Negative Control siRNA 1027280 (QIAGEN)

References

1. Wang Y, Huang S, Sah VP, Ross J, Jr., Brown JH, Han J, Chien KR. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem.* 1998;273:2161-2168.

2. Zhao W, Hirose T, Ishikawa M, Oshima Y, Hirai S, Ohno S, Taniguchi H. Neonatal pancreatic cells redifferentiate into both neural and pancreatic lineages. *Biochem Biophys Res Commun.* 2007;352:84-90.

3. Chiba T, Zheng YW, Kita K, Yokosuka O, Saisho H, Onodera M, Miyoshi H, Nakano M, Zen Y, Nakanuma Y, Nakauchi H, Iwama A, Taniguchi H. Enhanced self-renewal capability in hepatic stem/progenitor cells drives cancer initiation. *Gastroenterology.* 2007;133:937-950.