SUPPLEMENTAL DATA

AIP1 prevents graft arteriosclerosis by inhibiting IFN-γ-dependent smooth muscle cell proliferation and intimal expansion

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EXPANDED MATERIALS AND METHODS

Mouse allograft and syngeneic graft transplantation model

All animal studies were approved by the Institutional Animal Care and Use Committees of Yale University. WT mice were of C57BL/6 origin and knockout animals were backcrossed to this strain for over 15 generations. For allograft model, segments of thoracic aorta from male, 4-5 week old WT (C57/BL6), AIP1-KO, IFN-γR-KO or AIP1-KO/ IFN-γR-KO mice were interposed into the abdominal aorta of female recipient, 8-12 week old WT using an end-to-end microsurgical anastomotic technique. For syngeneic graft model, segments of thoracic aortae from male, 4-5 week old WT or AIP1-KO mice were interposed into the abdominal aortae of male, 8-12 week old IFN-γR1-KO using an end-to-end microsurgical anastomotic technique. At 1 week postoperatively, the animals were inoculated i.v. with Ad5.CMV-mouse IFN-γ or Ad5.CMV-LacZ (Qbiogene) at 1 x 10^9 PFU. Serum mouse IFN-γ levels were measured by ELISA (eBioscience) at 1 and 5 weeks after adenovirus administration. Certain animals received BrdU (Sigma-Aldrich) at 100 mg/kg s.c. for 2 weeks before sacrifice.

Graft analysis

Allografts were harvested at 4 weeks and syngrafts were at 6 weeks post-operatively (5 weeks after viral infection) and analyzed by standard histological techniques for Elastica-van Gieson (EVG) staining, hematoxylin and eosin (HE) staining and immunofluorescence staining using antibodies to mouse CD45 (BD Biosciences), mouse α-SMA (Sigma-Aldrich), BrdU (Abcam) and phospho-JAK2 (Cell Signaling Technology), followed by secondary antibody labeled with fluorochrome (Invitrogen). Images were captured using an immunofluorescence microscope system (Zeiss). Cell counting of nuclei surrounded by positive immunostaining was performed under high magnification and averaged from 5 cross-sections for each graft. Graft area measurements of the lumen (within the endothelium), intima (between the endothelium and internal elastic lamina, IEL), media (between the IEL and external elastic lamina, EEL), wall thickness (between the endothelium and external elastic lamina) and whole vessel (within the EEL) were calculated from 5 serial crosssections, 150 μm apart for each graft, using computer-assisted image analysis and NIH Image 1.60 (http://rsbweb.nih.gov/nih-image/)

Tissue and cell culture, inhibitors, cytokines, and transfection

Aorttic organ culture was performed as follows. Mice were anesthetized with ketamine and xylazine and sacrificed to isolate thoracic artery freshly. After removing the loose adhering adventitia, thoractc artery was washed in PBS and cut into small rings around 2-3 mm long. Then, aorta rings were incubated in DMEM (GIBCO) for 24 hours and treated with cytokines as indicated. Isolation of mouse aortic VSMC

was performed as follows. The aortas were excised, washed in PBS and the periadventitial fat and connective tissue were removed by microdissection followed by incubation in collagenase type A (Sigma-Aldrich) in PBS at 37°C for 10 to 15 minutes to remove endothelial cells. Under microscopic guidance, the remaining adventitia was removed with fine forceps. After incubating in complete culture medium containing DMEM supplemented with 10% FBS overnight, aorta fragments were digested with 2mg/ml collagenase type A and 0.5mg/ml elastase (Worthington) at 37°C for 30 minutes by pipetting every 5-10 minutes. Once the digestion was stopped by complete culture medium, cells were collected by spin down at 1000 *rpm* for 10 minutes and seeded in gelatin-coated culture dishes. Cultured cells exhibited typical spindle-shaped morphology of VSMCs and were verified to be VSMCs by immunostaining with α-SMA antibody (Sigma-Aldrich). Human aortic VSMCs were isolated by explant outgrowth from minced aorta or coronary arteries and cultured in supplemented M199 media with 20% FBS as previously described [1, 2]. For all experiments reported in this study, only passages 3 to 5 of primary cultured cells were used. Mouse recombinant IFN-γ, IFN-β, human recombinant IFN-γ, IFN-β were from Peprotech Inc. and PDGF-BB was from R&D Systems and were all used at 10 ng/ml. JAK2 inhibitor AG490 and STAT3 inhibitor Stattic were purchased from Calbiochem and STAT1 inhibitor fludarabine was purchased from Sigma-Aldrich. Transfection of 293T cells was performed by Lipofectamine according to the manufacturer's protocol (Invitrogen). Cells were cultured at 90% confluence in 6-well plates and were transfected with total 4 μg plasmid constructs as indicated. Cells were treated and harvested at 36–48 hours after transfection, and cell lysates were used for protein assays. AIP1 siRNA knockdown was performed as described previously [3, 4], modified from the manufacturer's protocol of Oligofectamine (Invitrogen). AIP1 siRNA (Ambion) was resolved to the concentration of 20μM. For cells in one well of 6-well plate, 2μl AIP1 siRNA and 8μl Oligofectamine were mixed in OPTIMEM I (GIBCO) sitting at room temperature for 30 minutes. VSMC were cultured at 90% confluence in 6-well plates and were transfected with the siRNA-Oligofectamine mixture in OPTIMEM I mentioned above for 12 hours followed by adding regular culture medium for the other 36 hours.

Immunoprecipitation and immunoblotting

Human VSMC or 293T cells transfectants after various treatments were washed twice with cold PBS and harvested in a membrane lysis buffer (30 mM Tris, pH 8.0, 10 mM NaCl,5 mM EDTA, 10 g/l polyoxyethylene-8-lauryl ether, 1 mM 0-phenanthroline,1 mM iodoacetamide, 10 mM NaF, 5 mM orthovanadate, and 10 mM sodium pyrophosphate). Cell lysates were then centrifuged at 14,000×*g* at 4°C for 15 minutes. Supernatants were used immediately for immunoblot or immunoprecipitation. To

analyze protein interaction in vivo via immunoprecipitation, supernatants of cell lysates were diluted 3 times with a cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.75% Brij 96, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM PMSF, and 1 mM EDTA). The lysates were then incubated with the first protein-specific antiserum (e.g., anti-AIP1) on ice for 1.5 hours. Then, 10 μl of protein A/G PLUS-agarose (Santa Cruz Biotechnology Inc.) was added and incubated for 2 hours with rotation. Immune complexes were collected after each immunoprecipitation by centrifugation at 13,000× *g* for 10 minutes followed by 3–5 washes with lysis buffer. The immune complexes were subjected to SDS-PAGE, followed by immunoblot with the second antibody (e.g.,JAK2). The chemiluminescence was detected using an ECL kit according to the instructions of the manufacturer (Amersham Life Science). For detection of FLAG-tagged proteins (AIP1), anti-FLAG M2 antibody (Sigma-Aldrich) was used for immunoblot. For immunoblot in culture aortae and culture cells, protein was extracted from homogenized tissues or cells in lysis buffer (1% NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 5mM EDTA, 1mM sodium orthovanadate, 0.1 % aprotinin, and 1 mM PMSF), and boiled in SDS sample buffer for 5 minutes. Equal amounts of protein per sample were separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene fluoride (Bio-Rad Laboratories). Antibodies against AIP1 were described previously [3]. Primary antibodies used included phospho-JAK1/2 (Biosource) phospho-JAK1, phospho-JAK2, phospho-Tyk2, phospho-STAT1, phospho-STAT3, phospho-AKT, phopho-PLCγ, phospho-PDGF receptor, JAK1, JAK2, Tyk2,STAT1, STAT3, AKT(Cell Signaling Technology) and β-actin (Sigma-Aldrich).

In vitro VSMC migration and proliferation assays

Monolayer VSMC migration. Monolayer VSMC migration was modified from the method described previously for vascular EC[5]. Briefly, human VSMC were cultured in serum-free medium for 24 hours followed by "wound injury" assay with a plastic pipette tip. Cells were plated with fresh media with or without IFN-γ and were further cultured for 36 hours. For inhibitor experiments, 25μM of AG490 (JAK2 inhibitor), Stattic (STAT3 inhibitor), fludarabine (STAT1 inhibitor) or AKT V (Akt inhibitor) were added (all inhibitors were purchased from Calbiochem). The VSMC migration in culture was determined by measuring wound areas in cell monolayers. Three different images from each well along the wound were captured by a digital camera under a microscope. Wound area (mm^2) was measured and analyzed by NIH Image 1.60 (http://rsbweb.nih.gov/nih-image/).

Transwell migration assay. Cell migration was examined using Transwell fitted with polycarbonate filters (8-μm pore size) (Corning Inc.). Briefly, the lower chambers were filled by M199 with or without

IFN-γ (10 ng/ml). Human VSMC were harvested in trypsin containing EDTA and were resuspended to a concentration of 1×10^6 cells/ml in M199. We loaded 1×10^5 human VSMC (0.1 ml) into each upper chamber and cells were cultured for 12 hours at 37°C in the presence of 5% CO_2 in a humidified incubator. Cells that did not migrate through the coated filters were removed with cotton swabs, and cells that migrated to the lower surfaces of the filters were stained for 30 minutes with 0.2% crystal violet in 10% ethanol. The chambers were washed thoroughly in a water reservoir, and the interior was dried with cotton swabs. Cells on the lower surfaces of the filters were counted under microscope.

Cell proliferation assay. Cell proliferation was assayed using a WST-1 Cell Proliferation Assay System (Roche Applied Science) according to the manufacturer's instructions. Briefly, control or AIP1 siRNA treated human VSMC were plated at a density of 5000 cells per well in a 48-well plate in 200 μL serum-free culture medium with 10ng/ml human IFN-γ, or with the addition of 25μM of AG490 (JAK2 inhibitor), Stattic (STAT3 inhibitor), fludarabine (STAT1 inhibitor) or AKTV (Akt inhibitor). To evaluate cell proliferation, cells were incubated for 1 to 3 days and subsequently exposed to 20 μl WST-1 reagent for 4 h at 37 °C in 5% CO_2. The absorbance of the treated samples against a blank control was measured at 450 nm as the detection wavelength and 670 nm as the reference wavelength for the assay.

Statistical analysis

All data are expressed as mean ± SEM. Two-tailed, paired t tests and a two-way ANOVA analysis were performed using the Prism software program (GraphPad Software). Differences with $P<0.05$ were considered to indicate statistical significance.

REFERENCES

1. Bai Y, Ahmad U, Wang Y, Li JH, Choy JC, Kim RW, Kirkiles-Smith N, Maher SE, Karras JG, Bennett CF, Bothwell AL, Pober JS, Tellides G. Interferon-gamma induces X-linked inhibitor of apoptosis-associated factor-1 and Noxa expression and potentiates human vascular smooth muscle cell apoptosis by STAT3 activation. *J Biol Chem.* 2008;283:6832-6842.
2. Wang Y, Bai Y, Qin L, Zhang P, Yi T, Teesdale SA, Zhao L, Pober JS, Tellides G. Interferon-gamma induces human vascular smooth muscle cell proliferation and intimal expansion by phosphatidylinositol 3-kinase dependent mammalian target of rapamycin raptor complex 1 activation. *Circ Res.* 2007;101:560-569.
3. Zhang R, He X, Liu W, Lu M, Hsieh JT, Min W. AIP1 mediates TNF-alpha-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. *J Clin Invest.* 2003;111:1933-1943.
4. Min W, Lin Y, Tang S, Yu L, Zhang H, Wan T, Luhn T, Fu H, Chen H. AIP1 recruits phosphatase PP2A to ASK1 in tumor necrosis factor-induced ASK1-JNK activation. *Circ Res.* 2008;102:840-848.
5. Luo D, Luo Y, He Y, Zhang H, Zhang R, Li X, Dobrucki WL, Sinusas AJ, Sessa WC, Min W. Differential functions of tumor necrosis factor receptor 1 and 2 signaling in ischemia-mediated arteriogenesis and angiogenesis. *Am J Pathol.* 2006;169:1886-1898.

Supplemental Figures

Fig.I. **Illustration and scheme of mouse allograft artery transplantation model**. An illustration (**A**) and scheme (**B**) of the allograft transplantation procedure. A segment of donor male thoracic aorta (WT or AIP1-KO) was surgically interposed into abdominal aorta in a WT female recipient. Aortas were harvested at 4 weeks post-transplantation. Histological analysis of artery grafts by Elastica–van Gieson (EVG), H&E and immunostaining. **C**. As a control, a segment of WT donor male thoracic aorta was surgically interposed into an abdominal aorta in a WT male recipient. No graft arteriosclerosis was observed. **D**. Expression of signature genes for Th1 (IFN-γ, TNF-α and T-bet) and Treg (TGF-β and Foxp3) were determined by qRT-PCR, and normalized to HPRT from WT and AIP1-KO grafts. Data are mean ± SEM from 6 mice per group. * P<0.05, ** P<0.001 comparing M to M *vs* M to F groups. ns: no significance comparing WT *vs* KO groups.

Fig.II. IFN-γ signaling is required for the minor antigen-driven allograft arteriosclerosis. Aorta from WT, IFN-γR-KO, AIP1-KO or AIP1-KO/IFN-γR-KO (DKO) mice was transplanted to female WT recipient. Aortas were harvested at 4 weeks post-transplantation. **A-B**. Histological analysis of artery grafts by Elastica–van Gieson (EVG), H&E and immunostaining with anti-α-SMA and anti-CD45. Representative photomicrographs are shown. Arrowheads mark the internal elastic lamina to delineate the intima from media. Scale bar: 50 μm. **C-D**. Morphometric assessment of artery graft intima, media, lumen and vessel area by computer-assisted microscopy and quantification of α-SMA$^+$ cells in intima and CD45$^+$ cells in intima and media for each artery graft. Data are mean ± SEM from 6 mice per group. * P<0.05, *** P<0.0001 comparing WT vs IFN-γR-KO groups, and AIP1-KO and DKO groups.

Fig.III. **IFN-γ-induced mouse syngeneic artery transplantation model**. **A**. A scheme of the IFN-γ-induced mouse syngeneic model. Male donor thoracic artery from WT or AIP1-KO was dissected and transplanted into abdominal aorta in male recipient IFN-γR KO mice. One week post surgery, 1X10^9 pfu Ad-mIFN-γ or Ad-LacZ was injected into recipient mice via jugular vein. Serum was collected on day 3, 7 and 35 post-injection of viruses for IFN-γ measurement. Grafts were harvested 6 weeks for morphometric assessment. **B**. Mouse IFN-γ in serum was measured by ELISA assay. Data are mean ± SEM from 6 mice per group. ns: no significance comparing WT *vs* KO groups.

Fig.IV. **Lack of inflammatory cells in IFN-γ-induced syngeneic vessel graft arteriosclerosis**. Male donor thoracic artery from WT or AIP1-KO was dissected and transplanted into abdominal aorta in male recipient IFN-γR KO mice. One week post surgery, 1X10^9 pfu Ad-mIFN-γ or Ad-LacZ was injected into recipient mice via jugular vein. Grafts were harvested 6 weeks after transplantation and VSMC and infiltration of immune cells were detected by immunostaining with anti-α-SMA and anti-CD45 antibodies. Representative photomicrographs are shown in **A** with quantifications in **B**. Arrowheads mark the internal elastic lamina to delineate the intima from

media. Data are mean±SEM from 6 mice in each group. ns: no significance comparing WT *vs* KO groups.

Fig.V. **No significant effects of AIP1 deletion on IFN-β signaling in VSMC**. **A**. WT and AIP1-KO mouse VSMCs were isolated (in Methods) and cultured in serum-free medium for 24 hours, followed by treatment with mouse IFN-β (10 ng/ml) for indicated times. Phosphorylation of Tyk2, and the total Tyk2, AIP1 and β-actin proteins were determined by Western blot with respective antibodies. **B**. Human VSMCs were transfected with a control or AIP1 siRNA for 48 hours followed by incubation in serum-free medium for 24 hours. Cells were treated with human IFN-β (10ng/ml) for indicated times. Phosphorylation of Tyk2, STAT1, and STAT3 and the total proteins were determined by Western blot with respective antibodies. All experiments were repeated at least three times.

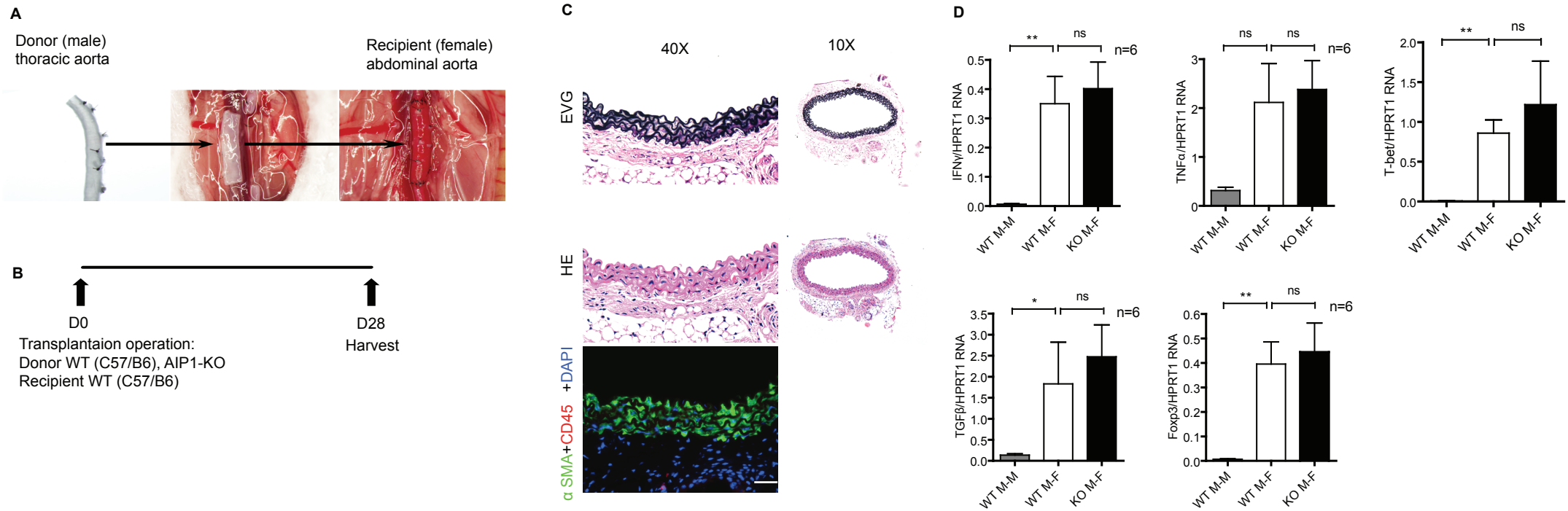
Fig.VI. Effects of AIP1 deletion and overexpression on IFN-γ-induced VSMC migration. Human VSMCs were transfected with AIP1 or control siRNA for 48 hours and cultured in serum-free medium for the other 24 hours. **A-B**. Cell migration in response to human IFN-γ (10ng/ml) was measured by a monolayer "wound injury" assay. Representative images are shown in **A**. Cell migration distances (mm) were quantified in **B**. **C-D**. IFN-γ treated cell were subjected to migration assay in the presence of JAK2, STAT3 or STAT1-specific inhibitor (AG490, Stattic or Fludarabine). Representative images for AIP1 siRNA group are shown in **C**. Cell migration distances (mm) were quantified in **D**. Data in B and D are mean±SEM from duplicates (10 different areas in each well) of three independent experiments. **E-F**. AIP1 overexpression reduces VSMC migration. Human VSMCs were infected with lentivirus expressing GFP or AIP1 for 48 hours and cultured in serum-free medium for the other 24 hours. Cell migration in response to human IFN-γ (10ng/ml) was measured by a monolayer "wound injury" assay. Representative images are shown in **E**. Cell migration distances (mm) were quantified in **F**. Data in B and D are mean±SEM from duplicates (10 different areas in each well) of three independent experiments. ***, $P<0.0001$ comparing lenti-AIP1 *vs* lenti-GFP. **G-H**. WT, AIP1-KO and AIP1/IFN-γR double KO (DKO) mouse VSMCs were isolated (in Methods) and cultured in serum-free medium for 24 hours. Cell migration in response to mouse IFN-γ (10ng/ml for 36 h) was measured by a monolayer "wound injury" assay. Representative images are shown in **G**. Cell migration distances (mm) were quantified in **H**. Data are mean±SEM from duplicates (10 different areas in each well) of three independent experiments. ***, $P<0.0001$ comparing AIP1-KO *vs* WT and DKO groups.

Fig.VII. No significant effects of AIP1 on PDGFβ production in graft and PDGFβ signaling in VSMC. Grafts from IFN-γ-induced mouse syngeneic artery transplantation model were harvested and transcript for PDGF-β in WT and AIP1-KO grafts was quantified by qRT-PCR and normalized to HPRT. Data are mean ± SEM from 5 mice per group. ns: no significance. **B**. Aortas dissected from WT and AIP1-KO mice were cultured overnight in serum-

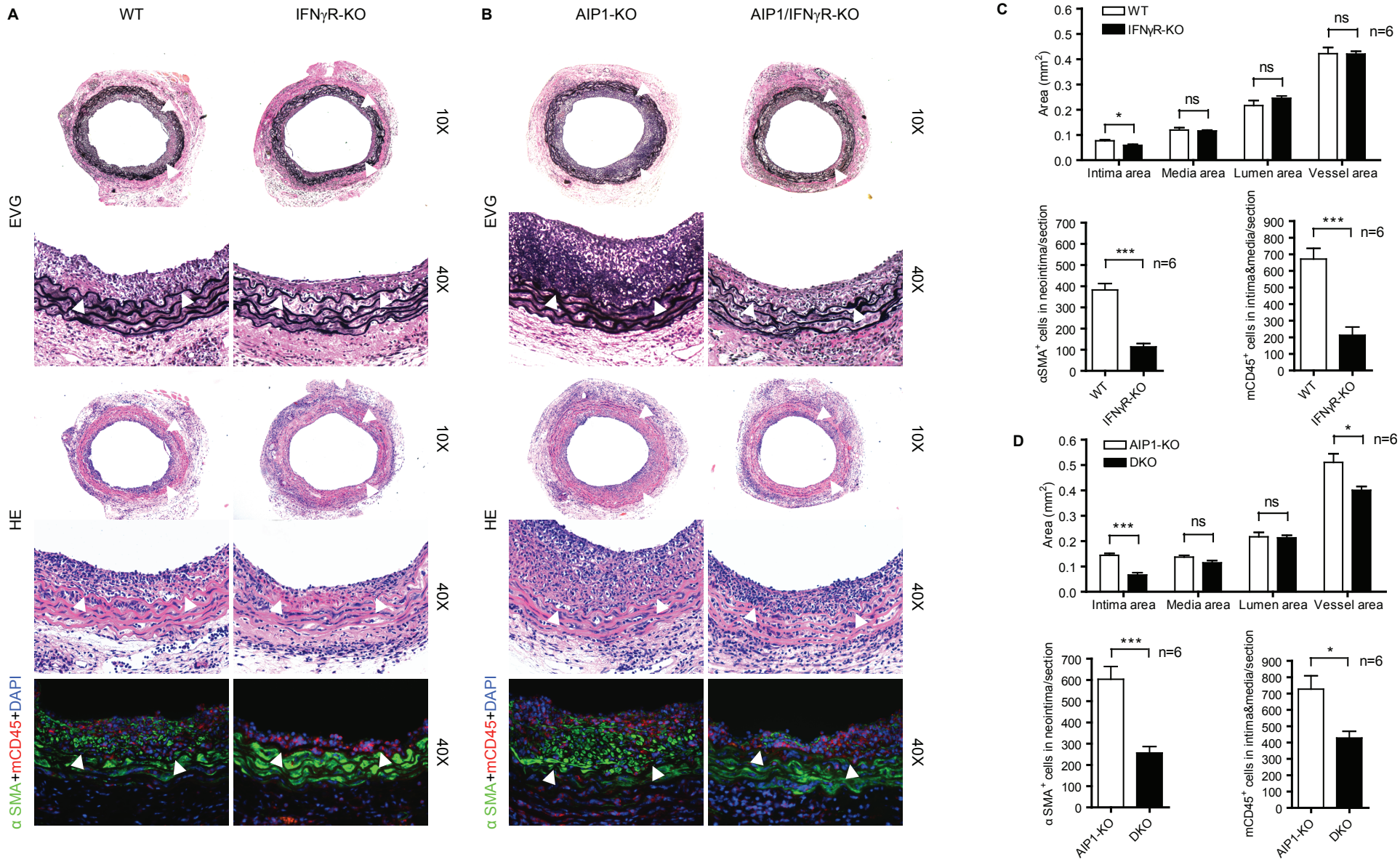
free medium (DMEM with penicillin/streptomycin), followed by treatment with PDGF-BB (10 ng/ml) for indicated times. Phosphorylation of PDGFRβ, PLC-γ and AKT, the total proteins were determined by Western blot with respective antibodies. **C**. Human VSMCs were transfected with a control or AIP1 siRNA for 48 hours followed by serum-starvation for 24 hours. Cells were treated with human PDGF-BB (10ng/ml) for indicated times. Phosphorylation of PDGFRβ, PLC-γ, AKT and JAK2 as well as the total proteins were determined by Western blot with respective antibodies. All experiments were repeated at least three times.

Fig.VIII. AIP1 deletion enhances IFN-γ-induced Akt activation and Akt-dependent SMC migration. Human VSMCs were transfected with AIP1 or control siRNA for 48 hours and cultured in serum-free medium for the other 24 hours. **A**. Cells were then treated with human IFN-γ (10 ng/ml) for indicated times. Phosphorylation of Akt, mTOR and the total proteins were determined by Western blot with respective antibodies. AIP1 and tubulin were also determined. Relative activation of Akt (p-Akt/Akt) and mTOR (p-mTOR/mTOR) is shown, with untreated Ctrl siRNA as 1.0. Experiments were repeated at least twice, and data are mean ± SEM from three independent blots. *P<0.05 comparing WT and AIP1-KO groups. **B-C**. Cells were subjected to migration assay in the presence of JAK2- or Akt-specific inhibitor (AG490 or AKTV). Representative images for AIP1 siRNA group are shown in **B**. Cell migration distances (mm) were quantified in **C**. Data are mean±SEM from duplicates (10 different areas in each well) of three independent experiments. **, P<0.001 and ***, P<0.0001 comparing IFN-γ-treated DMSO *vs* inhibitor groups.

Supplemental Fig.I

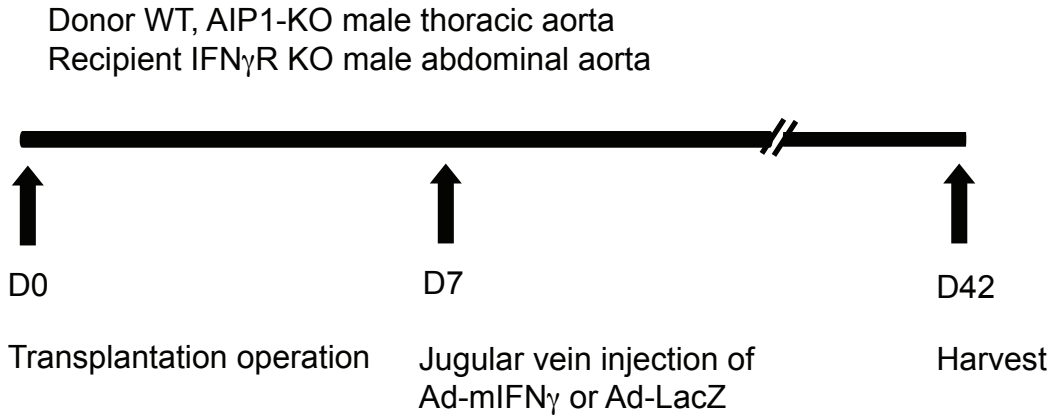


Supplemental Fig.II

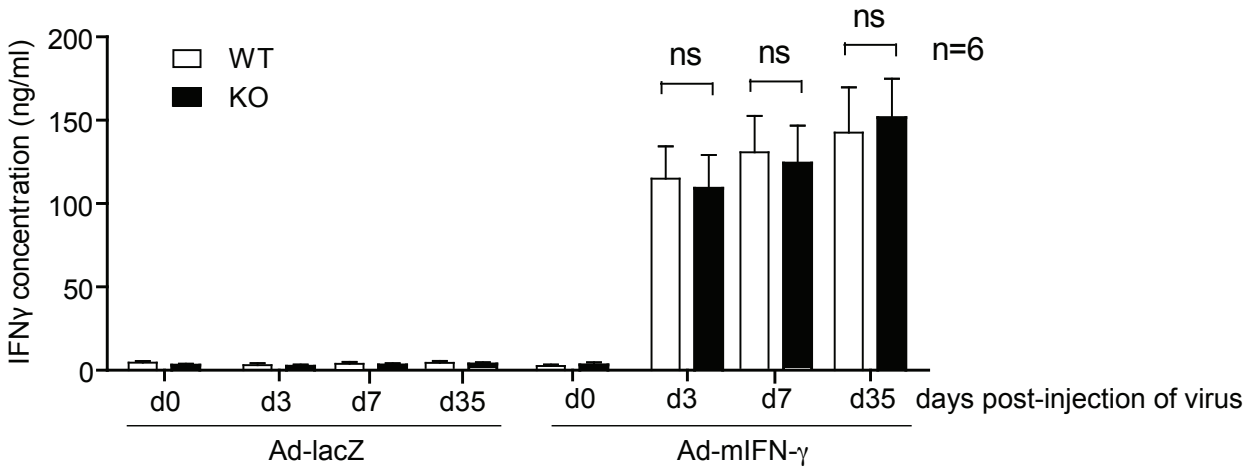


Supplemental Fig.III

A. IFNγ-mediated syngeneic mouse artery transplantation model



B. Kinetics of IFNγ expression in serum



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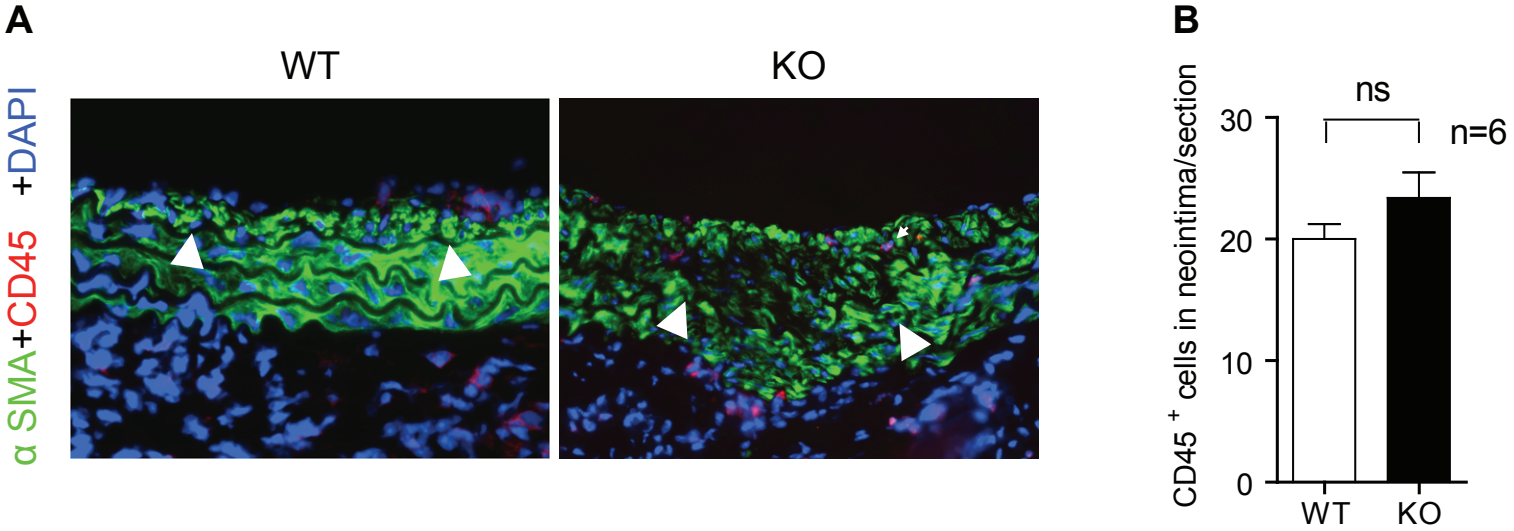

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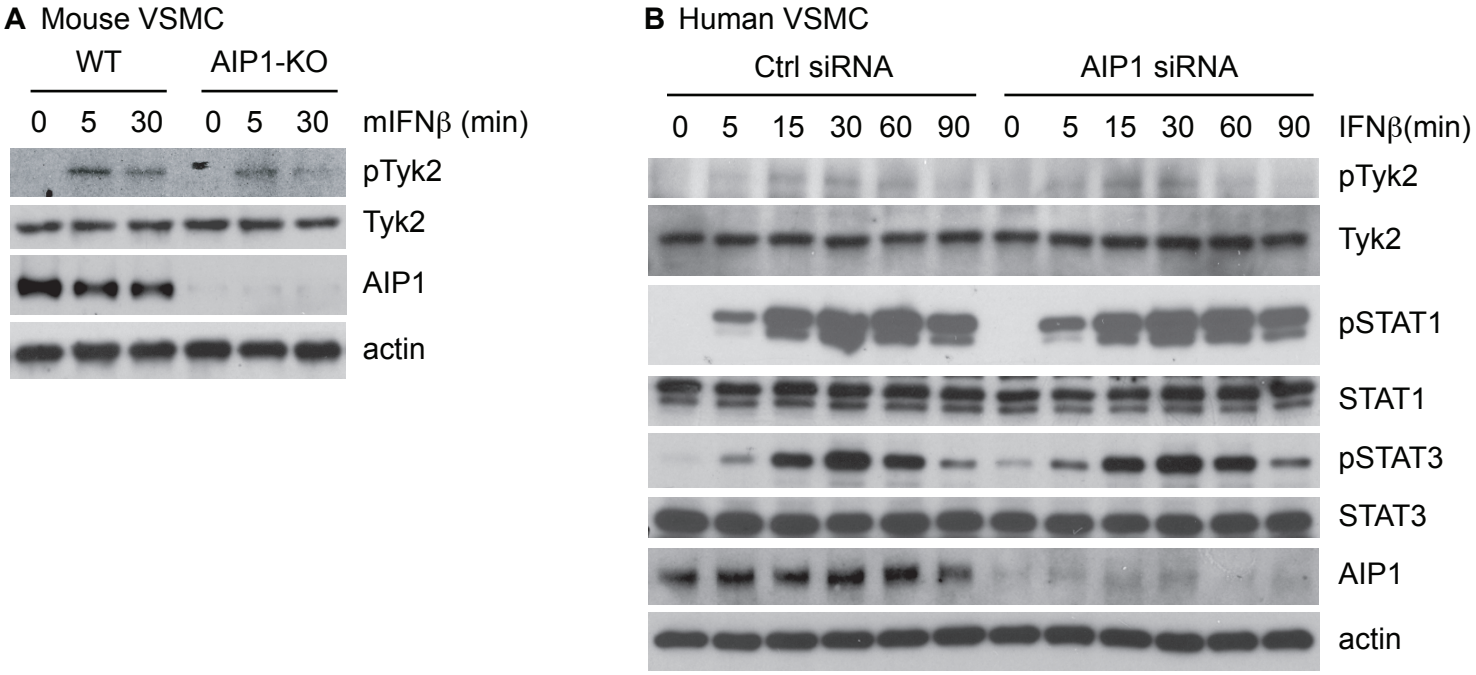

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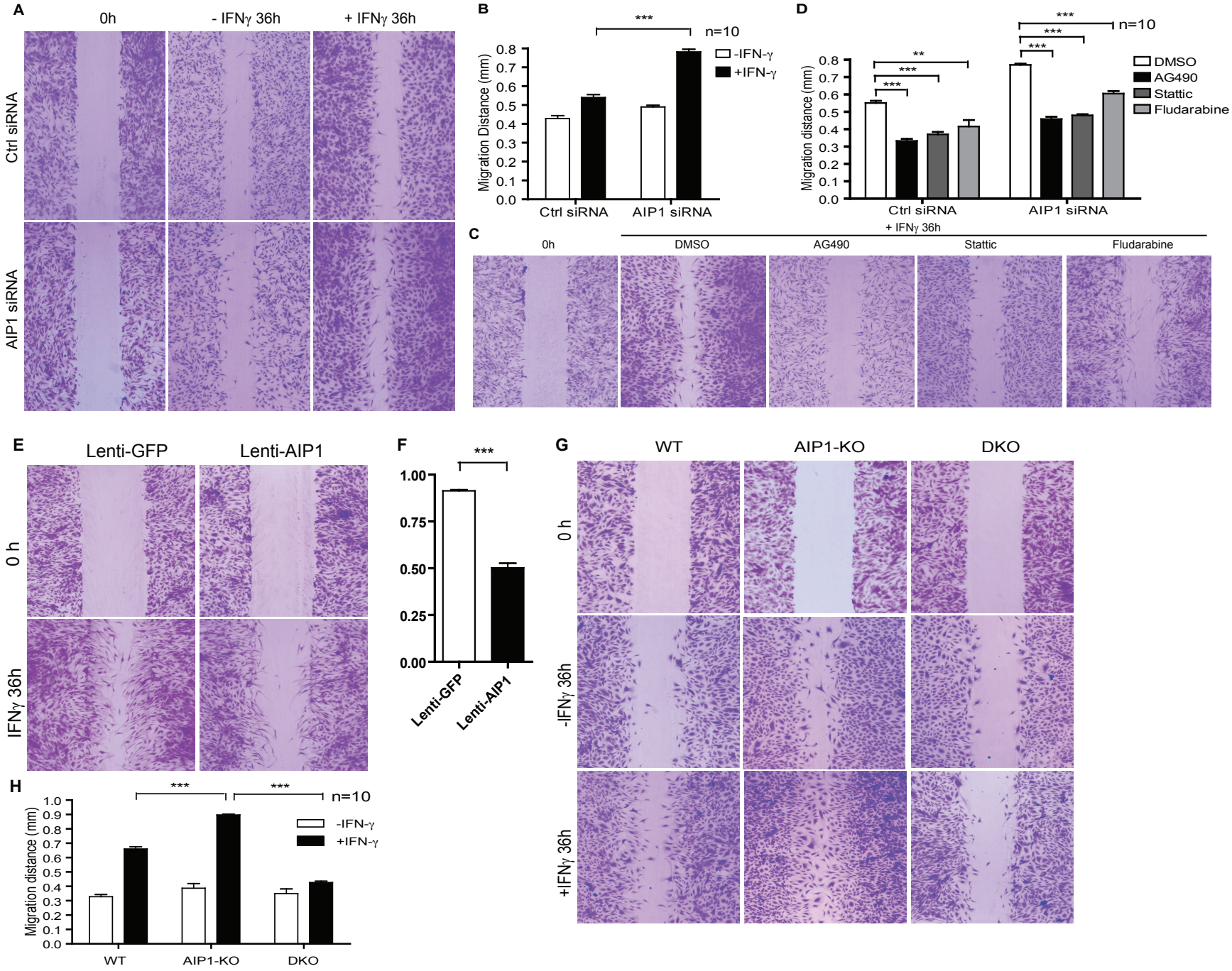


Supplemental Fig.VII

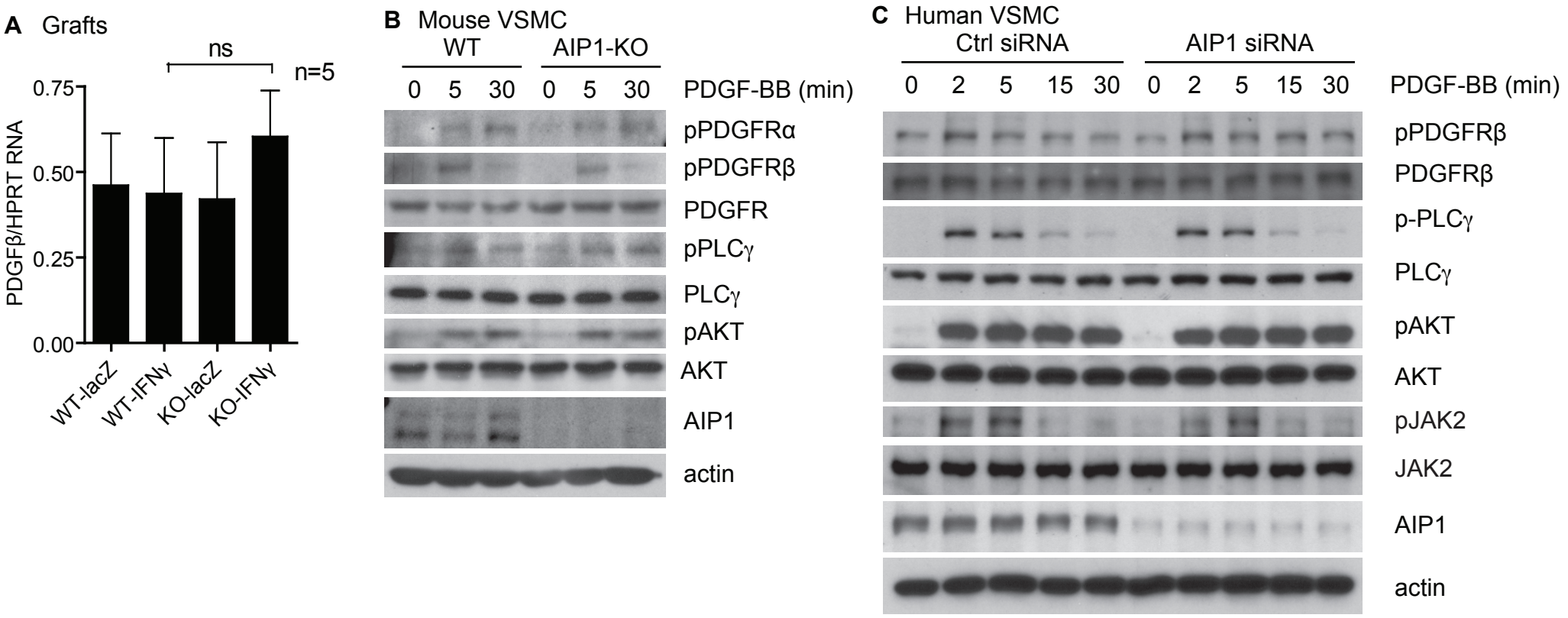
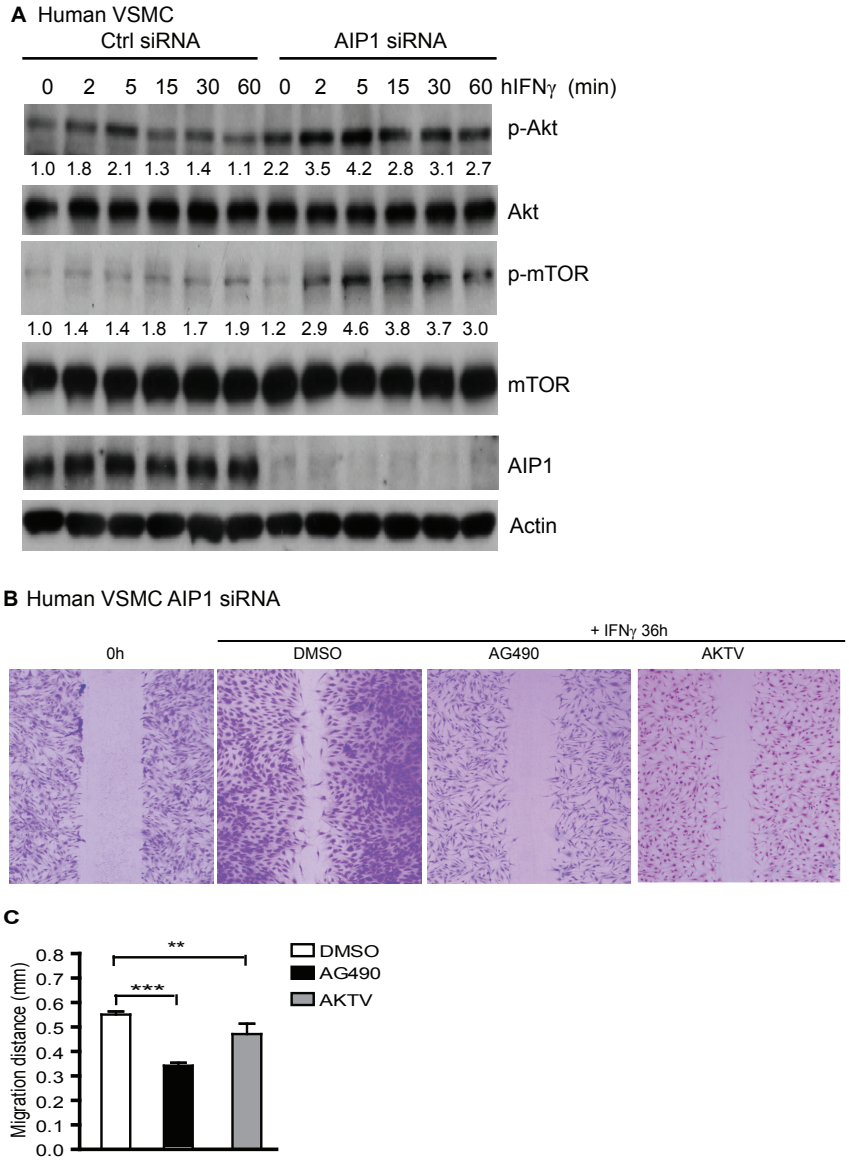


Fig.VIII


A Human VSMC
Ctrl siRNA
AIP1 siRNA
0 2 5 15 30 60 0 2 5 15 30 60 hIFNγ (min)
p-Akt
1.0 1.8 2.1 1.3 1.4 1.1 2.2 3.5 4.2 2.8 3.1 2.7
Akt
p-mTOR
1.0 1.4 1.4 1.8 1.7 1.9 1.2 2.9 4.6 3.8 3.7 3.0
mTOR
AIP1
Actin
B Human VSMC AIP1 siRNA
+ IFNγ 36h
0h
DMSO
AG490
AKTV
C
Migration distance (mm)
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
**
***
DMSO
AG490
AKTV