

Supporting Information

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SI Materials and Methods

Materials. Antibody for FLAG-tag was obtained from Stratagene. Antibodies for phospho-ERK, phospho-Akt, I κ B- α , survivin, and XIAP were obtained from Cell Signaling. Antibody for phospho-JNK was obtained from Promega. Antibodies for Bcl-2, Bcl-x, and CD31 were obtained from BD Biosciences Pharmingen. Antibodies for α -tubulin, TRAF-2, p53, P21^{WAF/CIP}, and cIAP-2 were obtained from Santa Cruz Biotechnology. Antibodies for cIAP-1 were obtained from Santa Cruz Biotechnology and BD Biosciences Pharmingen. Antibody for HA-tag was obtained from Roche Applied Science.

Quantitative RT-PCR. Total RNA was extracted from cells by using TRIzol (Invitrogen). When extracted from the *in vivo* samples, RNA was additionally purified by using RNeasy MiniElute cleanup kit (Invitrogen). cDNA was synthesized from 2.5 μ g of total RNA by using the SuperScript first-strand synthesis system (Invitrogen). PCRs were prepared by using LightCycler Fast-Start DNA Masterplus SYBR Green I (Roche Applied Science) followed by real-time PCR using LightCycler (Roche Applied Science). Expression of target genes was all normalized to GAPDH expression.

Generation of Anti-ARIA Antibody. A mixture of three epitopes for mouse ARIA (CRKNKESEDPOKPGS, ESCSTANGKDSITL, and CKGSMSAEKIL) was injected into a guinea pig. The antibody was prepared by affinity purification from the anti-serum.

Immunoprecipitation. For immunoprecipitation, cells were lysed with CellLytic lysis reagent (Sigma–Aldrich) and incubated with the antibody for the target protein at 4 °C overnight after the precleaning with protein G. Then, the target protein was immunoprecipitated by using protein G and subjected to SDS/PAGE.

Immunocytochemistry and Immunohistochemistry. For immunocytochemistry, cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. After blocking with 10% normal goat serum, cells were incubated with antibody for the target protein for 1 h at room temperature followed by incubation with secondary antibody labeled with the fluorescent dye.

For immunohistochemistry, mouse lung and spleen were fixed with 4% paraformaldehyde and embedded in paraffin. After blocking with methanol and 10% normal goat serum, sections were incubated with 1 μ g/ml anti-ARIA IgG for 1 h at room temperature followed by incubation with HRP-anti-guinea pig IgG antibody. The same concentration of normal guinea pig IgG was used as a negative control. Signals were visualized by using a DAB enzyme kit (Nichirei).

Cell Culture and Transfection. Full-length human and mouse ARIA was obtained by RT-PCR using Pfx DNA polymerase followed by subcloning into pCR blunt II-TOPO vector (Invitrogen). A BamHI site was created before the stop codon by adding 5'-GGATCC-3' at the beginning of reverse primer and the nucleotide sequence was validated. Fragments cut out with EcoRI and BamHI were subcloned into p3XFLAG-CMV-14 expression vector (Sigma–Aldrich) to obtain the expression constructs. Transfection of these expression constructs into

HeLa cells and bovine aortic endothelial cells was performed by using Lipofectamine 2000 (Invitrogen). For transfection into HUVECs, ARIA cDNA and FLAG-tag was cut out and subcloned into pMSCVneo vector (Clontech) to generate retrovirus. Human lung microvascular endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 MV medium (Clonetics) and HuMedia EG2 medium (Takara Bio), respectively, on gelatin-coated dishes.

Short interfering RNA (siRNA) was transfected into HUVECs mostly by electroporation. Negative control siRNA #1 (Ambion) was used as a control (scramble). Cells were mixed with 2.5 μ M siRNA in 75 μ L of siPORT siRNA electroporation buffer (Ambion) followed by electroporation at 250 V for 0.15 msec by square pulse. After incubation at 37 °C for 15 min, cells were transferred into fibronectin-coated dishes and cultured in the growth medium for 48–72 h. In some experiments, 10 nM siRNA was transfected by using RNAiMAX reagent (Invitrogen). siRNAs for cIAP-1 and cIAP-2 were obtained from Dharmacon. For apoptosis analysis, cells were replated onto fibronectin-coated 96-well plates and incubated overnight followed by incubation in serum- and growth-factor-depleted medium for 24 h. TUNEL staining was performed by using *in situ* cell death detection kit (Roche Applied Science). Cell migration was analyzed by modified Boyden chamber assay using 50 ng/ml VEGF as a chemoattractant as previously described. Cell proliferation was analyzed by using CellTiter 96 aqueous one solution cell proliferation assay kit (Promega) by culturing cells in the growth medium for 3 days.

Yeast Two-Hybrid Screening. Yeast two-hybrid screening was performed by using Matchmaker library construction and screening kit (Clontech). The C-terminal 61 aa of human ARIA fused with GAL4 DNA-binding domain was used as bait, and HUVEC cDNA library fused to the GAL4 activation domain was used as a prey. Expression constructs for the bait and prey were co-transformed into AH109 competent yeast cells followed by plating on quadruple-dropout SD agar plates (SD/–Ade/–His/–Leu/–Trp). After incubation at 30 °C for 1 week, positive colonies were picked up and screened by PCR. PCR products were all subjected to DNA sequencing.

In Vitro and in Vivo Angiogenesis Analysis Using Matrigel. *In vitro* angiogenesis analysis was performed in 96-well plates coated with 50 μ L of Matrigel (BD Biosciences). Two \times 10⁴ HUVECs were plated on Matrigel in the growth medium and incubated for 12–96 h. Number and length of tubes were counted in 4 independent fields. For *in vivo* Matrigel-plug assay, Matrigel was mixed with 50 ng/mL VEGF, 50 ng/mL basic FGF, and 50 units/mL heparin in the presence of either scramble siRNA (Ambion) or siRNA targeting mouse ARIA at 2 μ M. A pair of control and ARIA-knockdown Matrigels were injected *s.c.* into the bilateral flank of an 8-week old C57BL/6J mouse on day 0, and the Matrigel plugs were extracted at day 8. Isolation of CD31-positive endothelial cells from the Matrigel plugs, and RT-PCR analysis were performed as previously described (1).

Mouse Ischemic Retinopathy Model. P7 mice neonates were exposed to 75 \pm 2% oxygen for 5 days (P7–P12) and then placed in room air for 5 days (P12–P17). One microgram of scramble or ARIA siRNA was injected intravitreally immediately after placing in the room air. Five days after exposure to room air (P17), mice were killed and their eyes were rapidly removed. For

whole-mount imaging, eyes were fixed in 4% paraformaldehyde. The retinas were dissected and stained with *Griffonia simplicifolia* isolectin B4-Alexa 594 (Molecular Probes). For quantitation of retinal neovascularization, frozen sections of eyes were prepared and histochemically stained with biotinylated *Griffonia simplicifolia* isolectin B4 (Vector Laboratories). Slides were incubated with avidin coupled to peroxidase (Vector Laboratories) and visualized by using DAB enzyme kit (Nichirei).

For quantitative analysis, sections roughly 50–60 μm apart were stained with lectin, and the areas of lectin-stained cells on the surface of the retinas were measured.

TUNEL staining of the frozen sections was performed by using an in situ cell death detection kit (Roche Applied Science).

Tumor Model. B16 melanoma cells were obtained from the Human Science Research Resources Bank, Japan. Tumor cells

were subcutaneously inoculated at 2.5×10^6 cells per 100 μL of PBS into the flank of female nude mice (BALB/c nu/nu, Clea Japan). Ten days after cell inoculation, 12 μg of either scramble or ARIA siRNA complexed with InvivoFectamine (Invitrogen) was injected intratumorally (day 0). siRNA and InvivoFectamine complex was prepared as the supplier recommended. Intratumoral injection of siRNA was performed on days 0, 4, and 8. Tumor size was measured by digital caliper, and tumor volume was calculated as $0.5 \times \text{length} \times (\text{width})^2$. Extracted tumor xenografts were subjected to sectioning or RNA extraction.

Statistics. Statistical analyses were performed with the Student *t* test or with the Mann–Whitney *U* test. Differences (mean \pm SD) were considered significant when *P* was <0.05 .

1. Skovseth DK, Veuger MJ, Sorensen DR, De Angelis PM, Haraldsen G (2005) Endostatin dramatically inhibits endothelial cell migration, vascular morphogenesis, and perivascular cell recruitment in vivo. *Blood* 105:1044–1051.

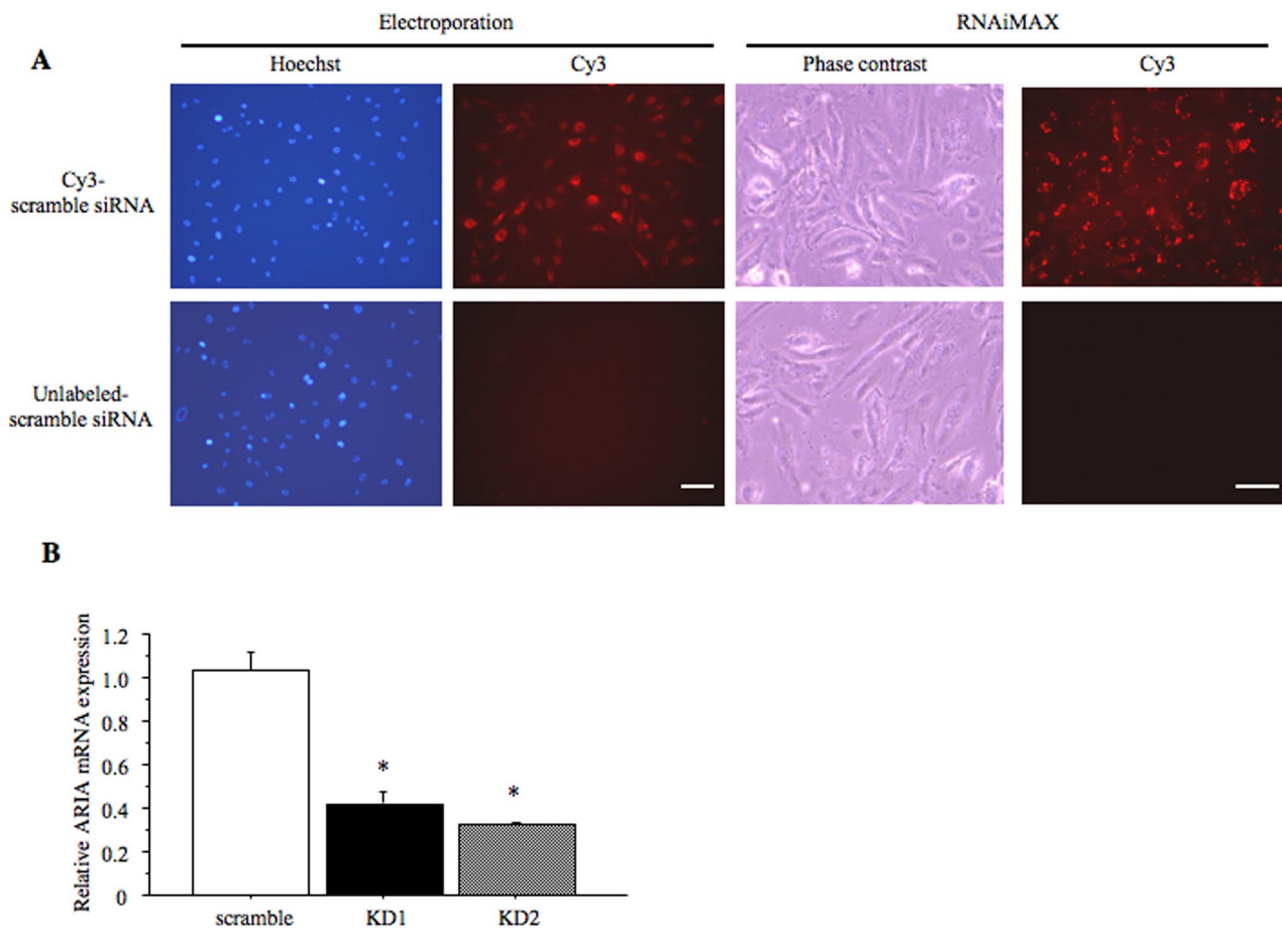


Fig. 51. Transfection of siRNAs into HUVECs. (A) Cy3-labeled or nonlabeled scramble siRNA was transfected into human umbilical vein endothelial cells (HUVECs) by electroporation or by using RNAiMAX reagent. After 24 h, cells were washed and the siRNA transfected into HUVECs was observed under a fluorescence microscope. (Scale bars, 100 μm .) (B) Short interfering RNAs were transfected into HUVECs by using RNAiMAX reagent. Expression of ARIA (apoptosis regulator through modulating IAPs expression) was quantitatively analyzed. *, $P < 0.01$ versus cells transfected with the scramble siRNA ($n = 3$ each).

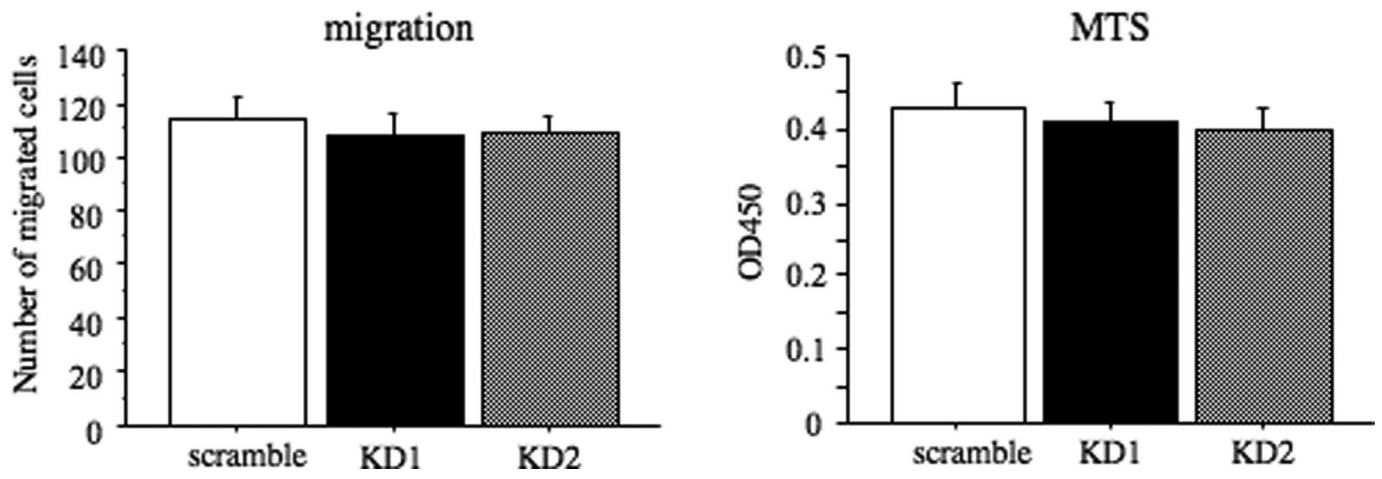


Fig. S2. ARIA-knockdown did not affect HUVEC migration or proliferation. Number of migrated cells was counted in an equal area ($n = 3$ each). Cell proliferation was assessed by the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay ($n = 4$ each).

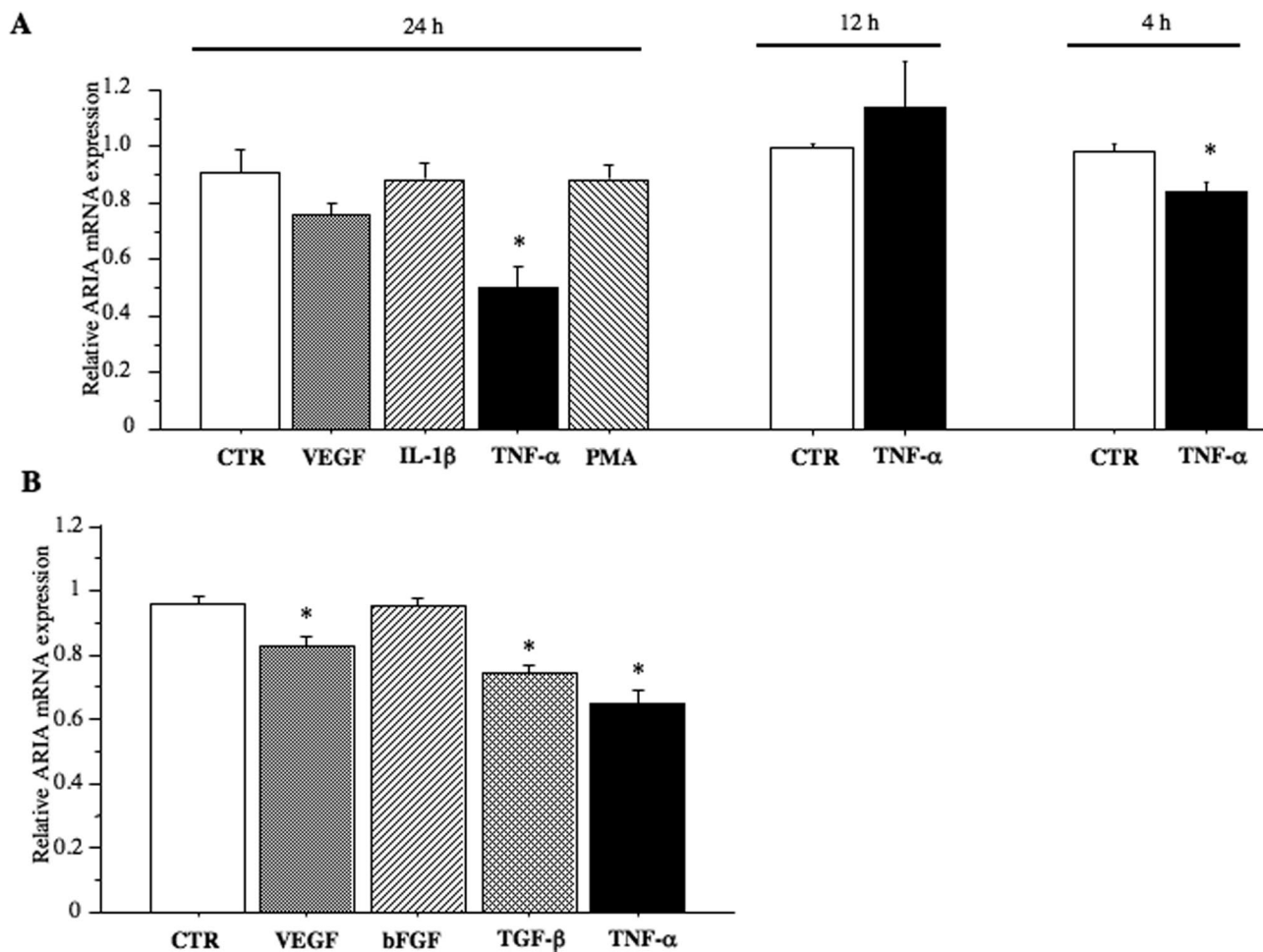


Fig. 53. Regulation of ARIA expression in endothelial cells. (*A*) human lung microvascular endothelial cells (HMVEC-lung) were stimulated with indicated stimulants for 24, 12, or 4 h, and then ARIA expression was examined by quantitative PCR. ARIA expression was significantly reduced by TNF- α . *, $P < 0.01$ versus cells treated with vehicle ($n = 3$ each). (*B*) HUVECs were stimulated with indicated stimulants for 24 h, and then ARIA expression was explored by quantitative PCR. ARIA expression was significantly reduced by VEGF, TNF- α , and TGF- β ($n = 3$ each). *, $P < 0.05$ versus cells treated with vehicle. Concentration of stimulant was 10 ng/mL for TNF- α , 1 ng/mL for TGF- β , 50 ng/mL for VEGF, 50 ng/mL for basic FGF, 10 units/mL IL-1 β , and 50 ng/mL for phorbol 12-myristate 13-acetate (PMA).

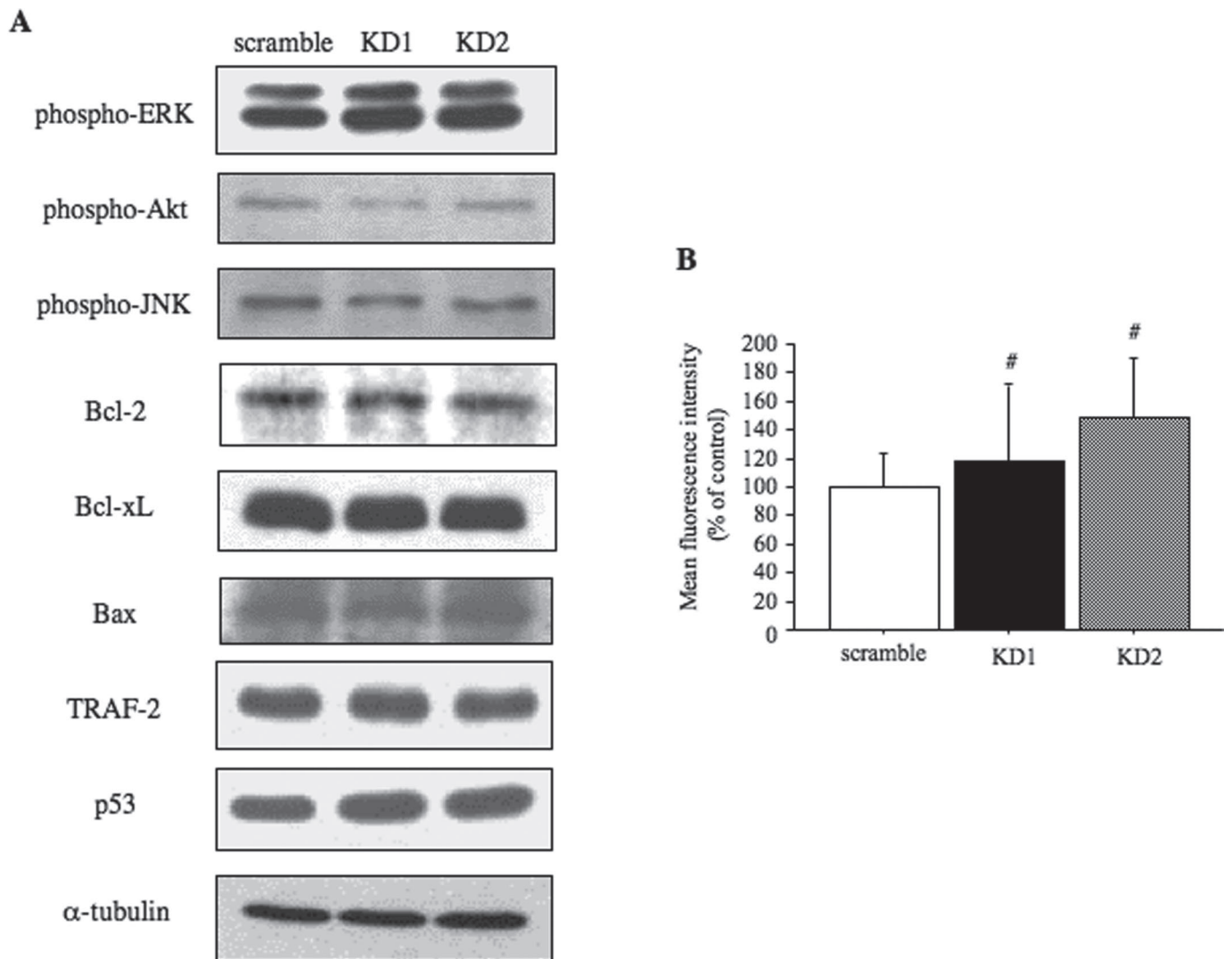


Fig. S4. Effect of ARIA knockdown on the apoptosis-associated factors. (A) Expression of factors associated with apoptosis such as phosphorylated MAPK or Akt, Bcl-2, Bcl-xL, Bax, TRAF-2, and p53 was not affected by ARIA- knockdown in HUVEC. (B) Reactive oxygen species production assessed by dichlorodihydrofluorescein (DCF) fluorescence was not affected by the ARIA-knockdown. #, NS versus the scramble control ($n = 4$ each).

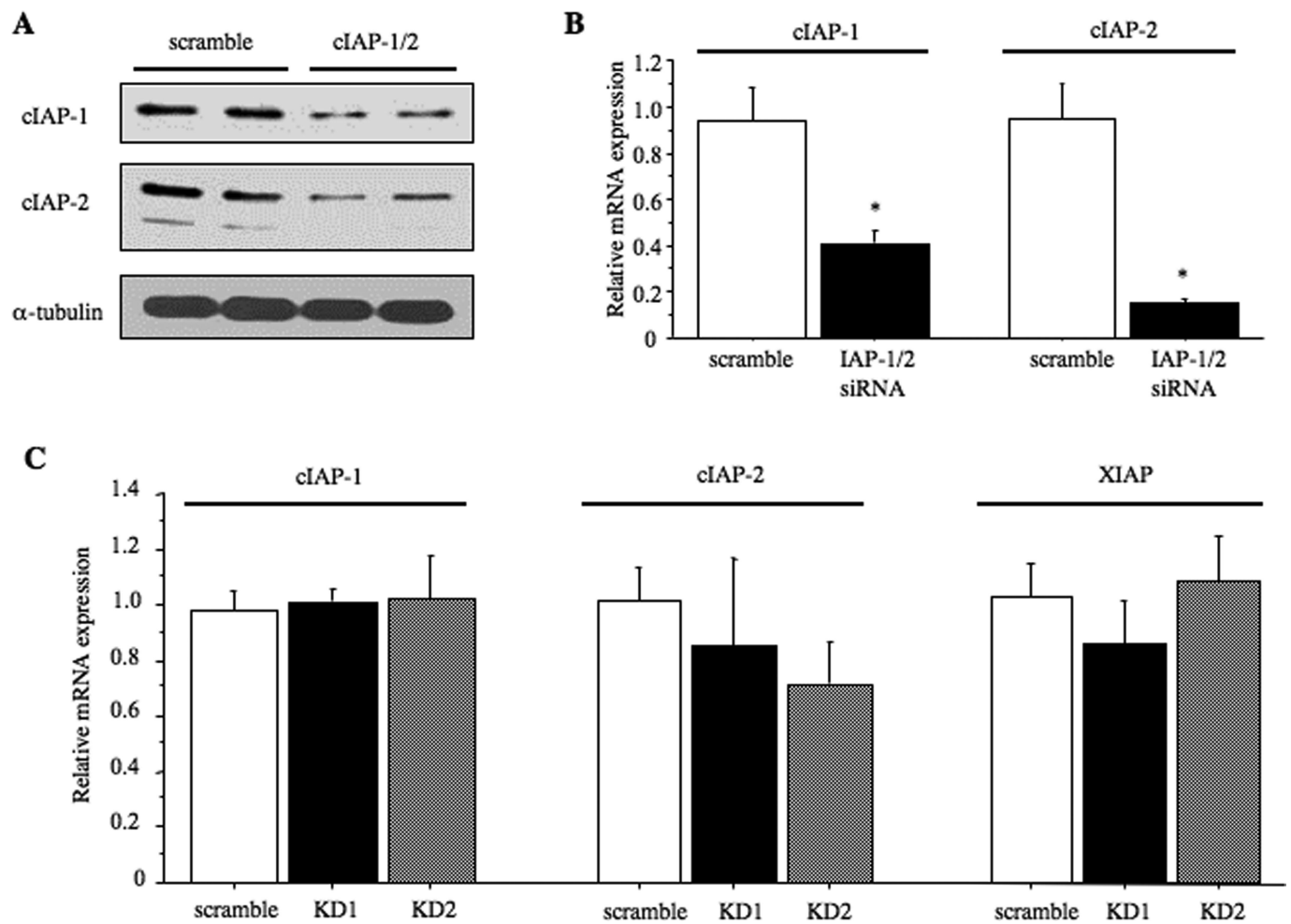


Fig. S5. Knockdown of the target genes. (A) Transfection of siRNA for cIAP-1 and cIAP-2 significantly knocked down their protein expression. (B) Transfection of siRNA for cIAP-1 and cIAP-2 significantly knocked down their mRNA expression. *, $P < 0.01$ versus cells transfected with the scramble siRNA ($n = 3$ each). (C) ARIA knockdown did not alter mRNA expression of cIAP-1, cIAP-2 and XIAP ($n = 4$ each).

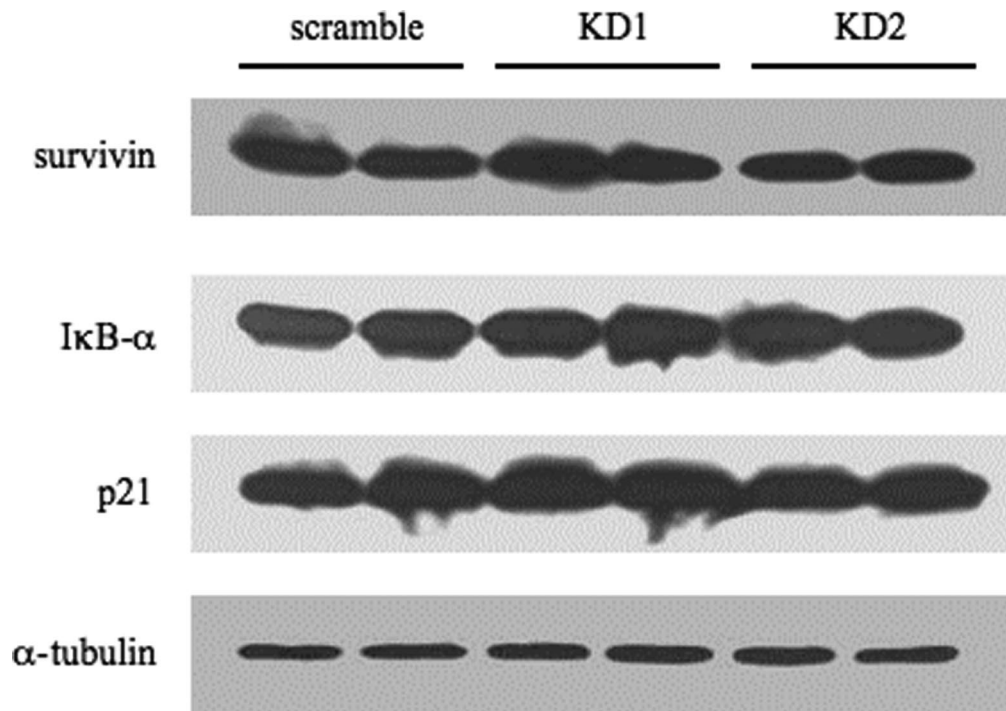


Fig. S6. ARIA knockdown did not affect the protein expression of survivin, I κ B- α , or p21^{WAF1/CIP}, which are targets for proteasomal degradation.

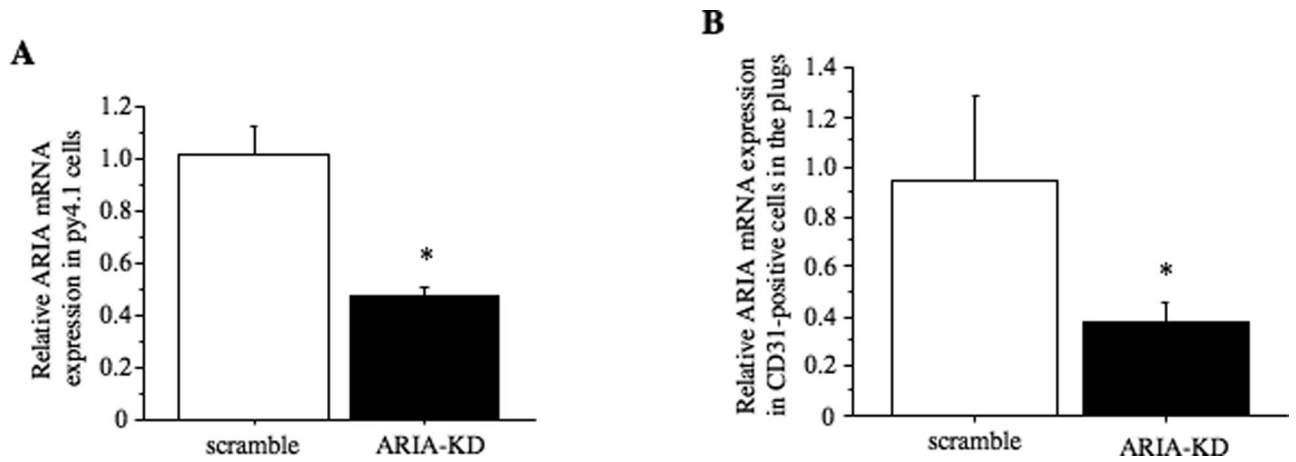


Fig. 57. Knockdown of ARIA. (A) Short interfering RNA targeting mouse ARIA was transfected into py4.1 mouse endothelial cells and ARIA mRNA expression was analyzed by quantitative PCR. *, $P < 0.01$ versus cells transfected with the scramble siRNA ($n = 3$ each). (B) Matrigel was prepared as described in *Materials and Methods* and injected s.c. into the mouse bilateral flank. CD31-positive endothelial cells were isolated from the Matrigel plugs and RNA was extracted. Knockdown of ARIA mRNA in CD31-positive endothelial cells was confirmed by quantitative PCR. *, $P < 0.05$ versus cells from the control plugs ($n = 4$ each).

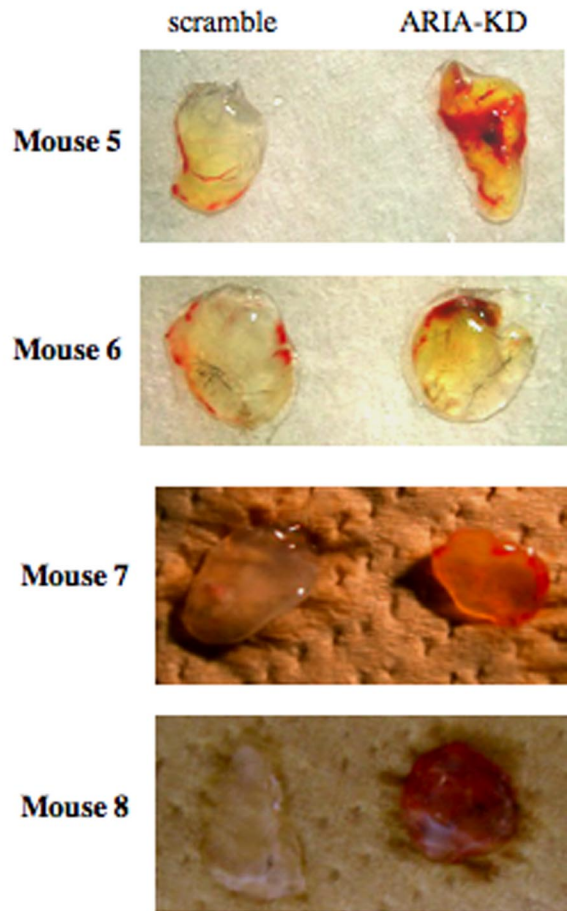
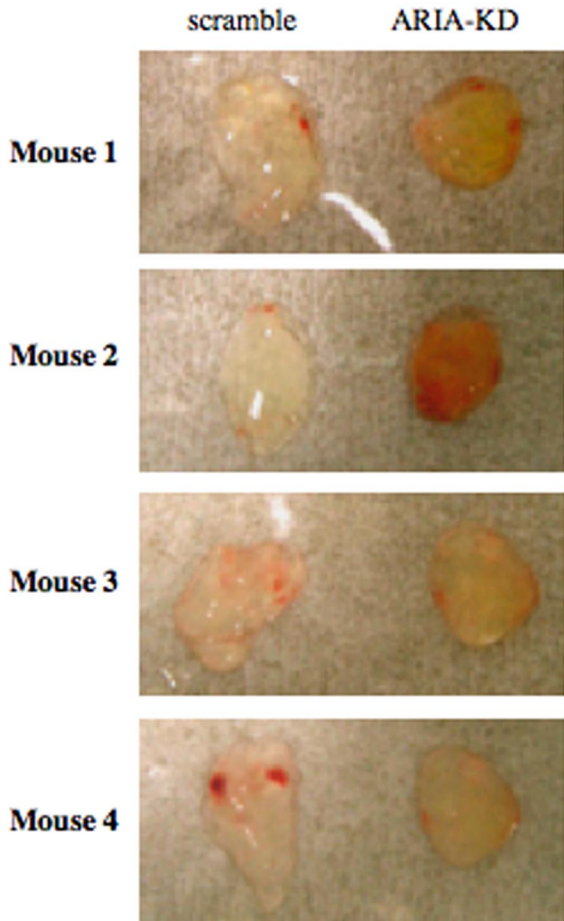
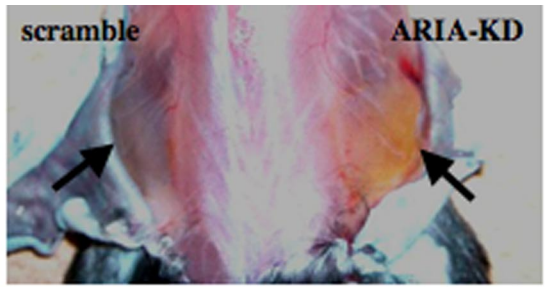


Fig. S8. A pair of Matrigels containing the scramble or ARIA siRNA were injected into the bilateral flank of one mouse at day 0 and then extracted at day 8. Appearance of the extracted plugs is shown.

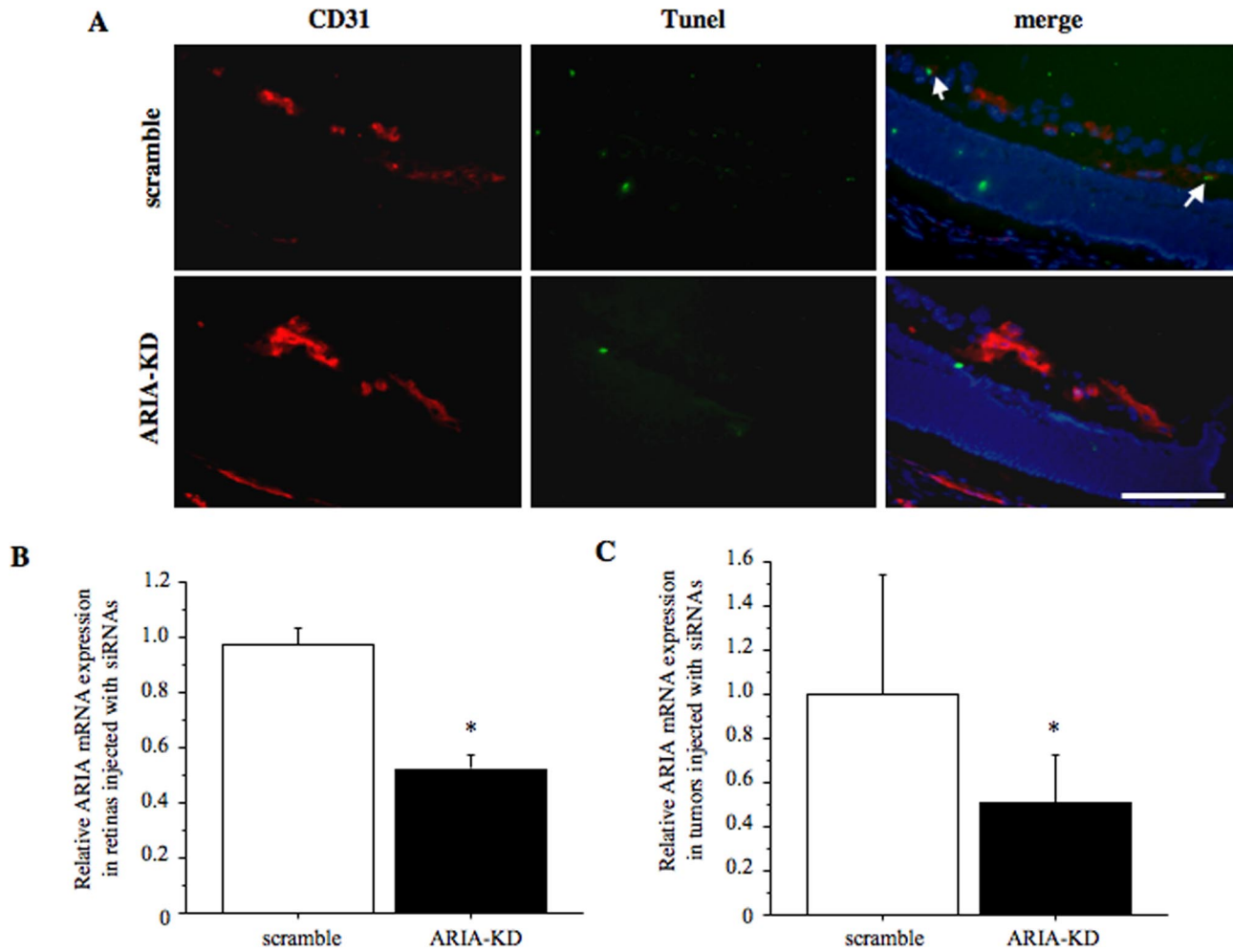


Fig. 59. Knockdown of ARIA in the retinas and tumor xenografts. (A) A small number of apoptotic endothelial cells that are double positive for TUNEL and CD31 (arrows) were observed at the surface of retinas injected with scramble siRNA. In contrast, apoptotic endothelial cells were rarely observed at the surface of retinas injected with ARIA siRNA. (Scale bar, 100 μ m.) (B) Either scramble or ARIA siRNA was injected intravitreally in neonatal mice with ischemic retinopathy. Expression of ARIA was significantly knocked down in the retinas injected with ARIA siRNA as compared with the scramble control. *, $P < 0.005$ versus the scramble control ($n = 6$ each). (C) Either scramble or ARIA siRNA was injected into tumor xenografts transplanted into the nude mice. Expression of ARIA was significantly knocked down in the tumor xenografts injected with ARIA siRNA as compared with the scramble control. *, $P < 0.05$ versus the scramble control ($n = 6$ each).