Apoptosis regulator through modulating IAP expression (ARIA) controls the PI3K/Akt pathway in endothelial and endothelial progenitor cells

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Endothelial and endothelial progenitor cells (ECs and EPCs) play a fundamental role in angiogenesis that is essential for numerous physiological and pathological processes. The phosphatase and tensin homolog (PTEN)/ phosphoinositide 3-kinase (PI3K) pathway has been implicated in angiogenesis, but the mechanism in the regulation of this pathway in ECs and EPCs is poorly understood. Here we show that ARIA (apoptosis regulator through modulating IAP expression), a transmembrane protein that we recently identified, regulates the PTEN/PI3K pathway in ECs and EPCs and controls developmental and postnatal angiogenesis in vivo. We found that ARIA is abundantly expressed in EPCs and regulates their angiogenic functions by modulating PI3K/Akt/endothelial nitric oxide synthase (eNOS) signaling. Genetic deletion of ARIA caused nonfatal bleeding during embryogenesis, in association with increased small vessel density and altered expression of various vascular growth factors including angiopoietins and VEGF receptors. Postnatal neovascularization induced by critical limb ischemia was substantially enhanced in ARIA-null mice, in conjunction with more bone marrow (BM)-derived ECs detected in ischemic muscles. Administration of PI3K or NO synthase inhibitor completely abolished the enhanced neovascularization in ARIA$^{-/-}$ mice. Mechanistically, we identified that ARIA interacts with PTEN at the intracellular domain independently of the PTEN phosphorylation in its C-terminal tail. Overexpressed ARIA increased PTEN in the membrane fraction, whereas ARIA-silencing reduced the membrane-associated PTEN, resulting in modified PI3K/Akt signaling. Taken together, our findings establish a previously undescribed mode of regulation of the PTEN/PI3K/Akt pathway by ARIA, and reveal a unique mechanism in the control of angiogenesis. These functions of ARIA might offer a unique therapeutic potential.

Angiogenesis occurs via the proliferation and sprouting of differentiated endothelial cells (ECs) from an existing vasculature, whereas postnatal vasculogenesis occurs by mobilization of bone marrow (BM)-resident endothelial progenitors into the peripheral blood circulation and their homing to sites of neovascularization. At the sites of neovascularization, endothelial progenitors are incorporated into growing vessels and differentiate into ECs to generate de novo blood vessels. Endothelial progeny differentiated from circulating progenitors are heterogeneous and can be divided into two types: early endothelial progenitor cells (early EPCs) and late outgrowth endothelial cells (late EPC or OECs) (1, 2). Early EPCs that show monocytic characteristics emerge early in the culture period and have poor proliferative capacity and highly express angiogenic cytokines stimulating angiogenesis in a paracrine manner (1, 3). In contrast, highly proliferative cells that show fully endothelial characteristics emerge late in the culture period. These cells are called late EPCs or OECs, and are considered to be bona fide BM-derived EPCs that contribute to vasculogenesis (4).

Angiogenesis and vasculogeneis are pivotally involved in various physiological and pathological processes such as wound healing, tumor growth, and ischemic diseases (5–11). The crucial role of EPCs in the pathogenesis of cardiovascular diseases has been well established (12) but the molecular mechanism regulating angiogenic functions in EPCs and/or OECs remain to be elucidated.

The PI3K/Akt signal is essential for angiogenesis through the regulation of various endothelial functions (13, 14), and is closely regulated by endogenous inhibitors including phosphatase and tensin homolog (PTEN). PTEN is one of the most important tumor suppressor genes, which is frequently mutated in human cancers, and is also involved in a diversity of biological processes such as neurogenesis, embryonic development, and angiogenesis (15–19). The regulation of the PTEN/PI3K pathway has been extensively studied in cancer cells, but the mechanism in the regulation of this pathway in ECs and EPCs is poorly understood. We recently identified a previously undescribed transmembrane protein, termed apoptosis regulator through modulating IAP expression (ARIA) that is highly expressed in ECs and regulates endothelial apoptosis by modulating the expression of inhibitor of apoptosis (cIAP)-1 and cIAP-2 (20). However, the role of ARIA in the vascular development and postnatal angiogenesis remained unclear. Here, we show that ARIA is abundantly expressed in EPCs and regulates their angiogenic functions through a previously undescribed PTEN-mediated mechanism. Furthermore, targeted deletion of ARIA revealed its role in the embryonic vascular development and postnatal ischemia-induced neovascularization, shedding light on ARIA as a potential pharmacotherapeutic target.

Results

ARIA Regulates Angiogenic Functions in EPCs by Modulating the PI3K/Akt/Endothelial Nitric Oxide Synthase (eNOS) Signaling. We previously found that ARIA is expressed in hematopoietic cells in addition to its high expression in ECs (20), which urged us to investigate whether ARIA is also expressed in EPCs. Quantitative PCR analysis revealed that early EPCs expressed *ARIA* in a comparable level to human umbilical vein endothelial cells (HUVECs), which we believed expressed ARIA the most (Fig. 1*A*). Of note, OECs isolated from human peripheral and cord blood abundantly expressed *ARIA*: 50- to 60-fold higher than early EPCs and 40- to 50-fold higher than HUVECs (Fig. 1*A*). We therefore examined the role of ARIA in the regulation of angiogenic functions in OECs. Short interfering RNA (siRNA)-mediated gene silencing of ARIA (Fig. S1*A*) substantially reduced apoptosis and enhanced tube formation on Matrigel in OECs isolated from peripheral blood (Fig. 1 *B* and *C*). ARIA silencing also attenuated apoptosis and accelerated the migration toward SDF1α in OECs isolated from cord blood (Fig. 1 *D*

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and *E*). Reexpression of ARIA by using retrovirus reversed these effects of ARIA silencing on apoptosis and migration, excluding the possibility of unspecific effects by silencing off-targeted genes (Fig. S1*B*).

Consistent with our previous report, ARIA-silencing significantly enhanced the expression of cIAP-1 and cIAP-2 in OECs (Fig. S1*C*). Nevertheless, simultaneous knockdown of cIAP-1 and cIAP-2 did not affect the enhanced migration of OECs in which ARIA was silenced (Fig. 1*F*). We therefore studied the effect of pharmacological inhibition of MAPK/ERK kinase (MEK), phosphoinositide 3-kinase (PI3K), and NO synthase (NOS), which play crucial roles in the regulation of angiogenesis on the enhanced OECs migration induced by ARIA silencing. Inhibition of NOS and PI3K significantly attenuated the OECs migration, whereas MEK inhibition did not (Fig. 1*G*). Of note, PI3K inhibition completely abolished the accelerated migration of OECs in which ARIA was silenced (Fig. 1*G*). Similarly, NOS inhibition substantially attenuated the accelerated migration of OECs by ARIA silencing, whereas MEK inhibitor showed minimal effect (Fig. 1*G*). In contrast, PI3K inhibitor failed to abrogate the reduced apoptosis by ARIA silencing, and a combination of PI3K inhibition and gene silencing of cIAP-1 and cIAP-2 was required to completely abolish the antiapoptotic effect of ARIA silencing (Fig. S1*D*). We then evaluated the activity of PI3K/Akt/eNOS signaling and found that these signals are significantly accentuated in OECs when ARIA was silenced (Fig. 1*H* and Fig. S2). These data collectively indicate that ARIA regulates angiogenic functions in EPCs by modulating the PI3K/Akt/eNOS signaling.

ARIA Controls Embryonic Vascular Development and Postnatal Neovascularization in Vivo. To investigate the in vivo function of ARIA in neovessel formation, we generated mice with a targeted deletion of ARIA (Fig. S3 *A*–*C*). Homozygote ARIA knockout mice were viable and fertile, but we found that ARIA$^{-/-}$ embryos demonstrated nonfatal bleeding without apparent developmental delay (Fig. 2 *A* and *B*). There was no significant abnormality of placenta in size and structure in ARIA$^{-/-}$ embryos (Fig. S3*D*). Gene expression analysis for vascular growth factors in the whole yolk sacs revealed enhanced mRNA expression of *angiopoietin-1* and *-2*, *Flt-1*, *kinase insert domain receptor* (*KDR*), *TGF-β1*, *TGF receptor-1*, and reduced expression of *VCAM-1* in ARIA$^{-/-}$ embryos (Fig. 2*C*). Histological analyses revealed that there was no significant difference in the pericytes or vascular smooth muscle cell coverage for large vessels, but the density and the number of small vessels less than 10 μm in diameter was increased in ARIA$^{-/-}$ embryos (Fig. 2 *D* and *E*). These strongly indicate that ARIA is involved in the embryonic vascular development.

We then investigated the role of ARIA in postnatal angiogenesis. Blood flow recovery in response to critical limb ischemia was substantially augmented, and the capillary density in ischemic adductor muscles was significantly increased in ARIA$^{-/-}$ mice (Fig. 3 *A* and *B*). As was observed in human OECs, ARIA was highly expressed in mouse BM-derived EPCs, whereas

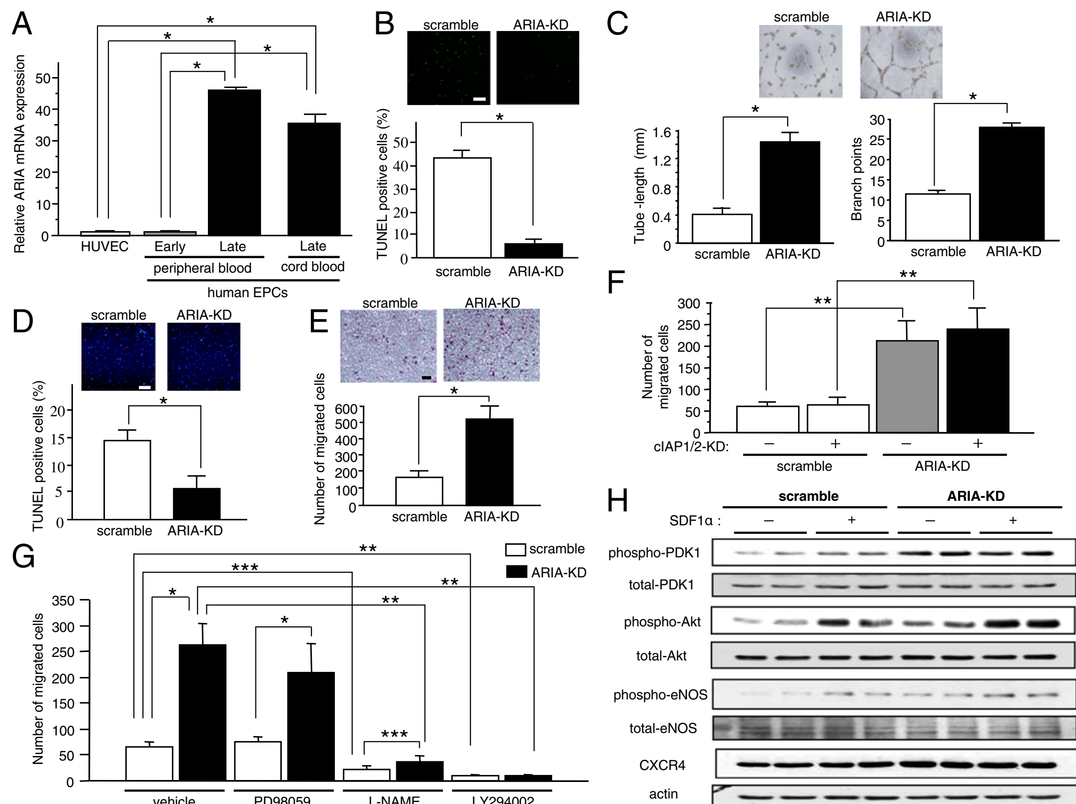

Fig. 1. ARIA regulates angiogenic functions in EPCs by modulating PI3K/Akt/eNOS signaling. (*A*) Quantitative real-time PCR of ARIA expression in HUVECs, early and late EPCs isolated from peripheral blood, and late EPCs isolated from cord blood (*n* = 4 each). (*B*–*E*) Apoptosis (*B*, TUNEL-positive cells, green) and tube formation (*C*) were examined in peripheral blood late EPCs (OECs) transfected with scramble or ARIA siRNA (ARIA KD). Apoptosis (*D*, TUNEL-positive cells, red and nuclei, blue) and migration toward SDF1α (*E*) were examined in cord blood OECs transfected with scramble or ARIA KD (*n* = 5 each). (*F*) Migration of OECs transfected with scramble or ARIA KD concomitantly with scramble or cIAP-1 and -2 siRNA (cIAP1/2-KD) (*n* = 5 each). (*G*) Migration of OECs transfected with scramble or ARIA KD. Cells were treated with vehicle, MEK inhibitor (PD98059), NOS inhibitor (L-NAME), or PI3K inhibitor (LY294002) (*n* = 5 each). (*H*) PI3K/Akt/eNOS signaling was examined by immunoblotting in OECs transfected with scramble or ARIA KD in the presence or absence of SDF-1α stimulation. Immunoblotting, in which two independently prepared samples were used, was repeated three times, and the representative results are shown. The quantitative data are shown in Fig. S2. Data are represented as mean ± SEM. *$P < 0.0001$, **$P < 0.01$, ***$P < 0.05$. (Scale bar, 100 μm.)

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modest expression was detected in BM cells, BM mononuclear cells, and BM Lin^{-}/c-kit^{+} cells, suggesting that ARIA expression might be induced during the differentiation of BM cells into endothelial progeny to regulate their angiogenic functions (Fig. S4*A*). We therefore generated BM-chimeric mice by transplanting the BM cells of ARIA$^{-/-}$/GFP^{+} or ARIA$^{+/+}$/GFP^{+} mice into wild-type or ARIA$^{-/-}$ mice to separately evaluate the contribution of ECs in existing blood vessels and BM-derived EPCs to the enhanced neovascularization in ARIA-null mice. BM cells were efficiently reconstituted by the donor cells, and there was no significant difference in the percentage of chimerism between the groups (Fig. S4*B*). Targeted deletion of ARIA in BM cells led to enhanced blood flow recovery in ischemic limbs and increased capillary density in ischemic muscles (Fig. 3 *C* and *D*). Mobilization of EPCs into the peripheral blood circulation in response to limb ischemia did not differ between wild-type and ARIA$^{-/-}$ mice (Fig. S4*C*). Nevertheless, more BM-derived ECs (GFP^{+}/isolectin^{+} cells) were detected in ischemic adductor muscle in mice having ARIA$^{-/-}$ BM cells (Fig. 3*E* and Fig. S4*D*). These results indicate a role for ARIA in postnatal vasculogenesis, probably through the regulation of BM-derived EPCs functions.

ARIA deletion in recipient mice also led to enhanced blood flow recovery in ischemic limbs and increased capillary density in ischemic adductor muscles compared with those in wild-type mice with the same genotype BM cells (Fig. 3 *C* and *D*). Unexpectedly, ARIA deletion in recipient mice also increased the number of BM-derived ECs in ischemic muscles (Fig. 3*E* and Fig. S4*D*). Activated Akt in ECs of ischemic lesion has been reported to play a crucial role in the homing and incorporation of EPCs into neovessels (21). In fact, genetic deletion of ARIA led to accelerated Akt signaling in aortic ECs (Fig. 4*A* and Fig. S5). Therefore, ARIA-deletion might enhance postnatal vasculogenesis in a dual pathway, accelerating angiogenic functions in EPCs and enhancing the homing of EPCs through augmenting Akt activation in ECs of ischemic lesion. Also, ECs isolated from ARIA$^{-/-}$ mice aorta showed reduced apoptosis and accelerated migration and tube formation, indicating a role for ARIA in angiogenesis through the regulation of ECs functions (Fig. 4*B*). Consistent with the crucial role of PI3K/Akt/eNOS signals in ARIA function in vitro, administration of PI3K or NOS inhibitors completely abolished the enhanced ischemia-induced neovascularization in ARIA$^{-/-}$ mice (Fig. 4 *C* and *D*). Together, ARIA controls postnatal angiogenesis and vasculogenesis by regulating the PI3K/Akt/eNOS signaling both in vitro and in vivo.

ARIA Controls PI3K/Akt Signals by Regulating the Membrane-Association of PTEN. To gain mechanistic insights into the regulation of PI3K/Akt signaling by ARIA, we studied the possible interaction of ARIA with molecules involved in the PI3K/Akt signals. We found a coprecipitation of endogenous PTEN with recombinant ARIA–FLAG expressed in py4.1 cells, an established mouse endothelial cell line that has been isolated from hemangioma arising in transgenic mice carrying the entire polyoma early genetic region (22) (Fig. 5*A*). The association between ARIA and PTEN was further confirmed by the coprecipitation of recombinant HA–PTEN and ARIA–FLAG (Fig. S6*A*). PTEN is a nonredundant, plasma membrane lipid phosphatase that antagonizes the PI3K function (23). Membrane association of PTEN is important as it dephosphorylates the target phospholipid on the plasma membrane to function (19, 24). ARIA is a transmembrane protein as we previously reported, and we detected PTEN colocalizing with ARIA at the plasma membrane (Fig. 5*B*). More importantly, stable expression of ARIA substantially increased the membrane-associated PTEN in CHO cells, which do not express ARIA endogenously, without affecting the total amount of PTEN (Fig. 5*C* and S6*B*). Conversely, siRNA-mediated ARIA silencing in human OECs and genetic deletion of ARIA in mouse aortic ECs considerably reduced the membrane-associated PTEN, whereas the total amount of PTEN was not altered (Fig. 5*C* and S6*B*). Moreover, stable ARIA expression in CHO cells reduced the phosphatidylinositol (3,4,5)-trisphosphate (PIP3), membrane phospholipids that are a PTEN substrate, strongly suggesting that ARIA enhances PTEN function by anchoring PTEN to the membrane (Fig. 5*D*).

Phosphorylation of PTEN at Ser and Thr in the C-terminal tail modulates its catalytic activity toward lipid substrates, due to changes in its ability to interact with membranes (25–27). Stable

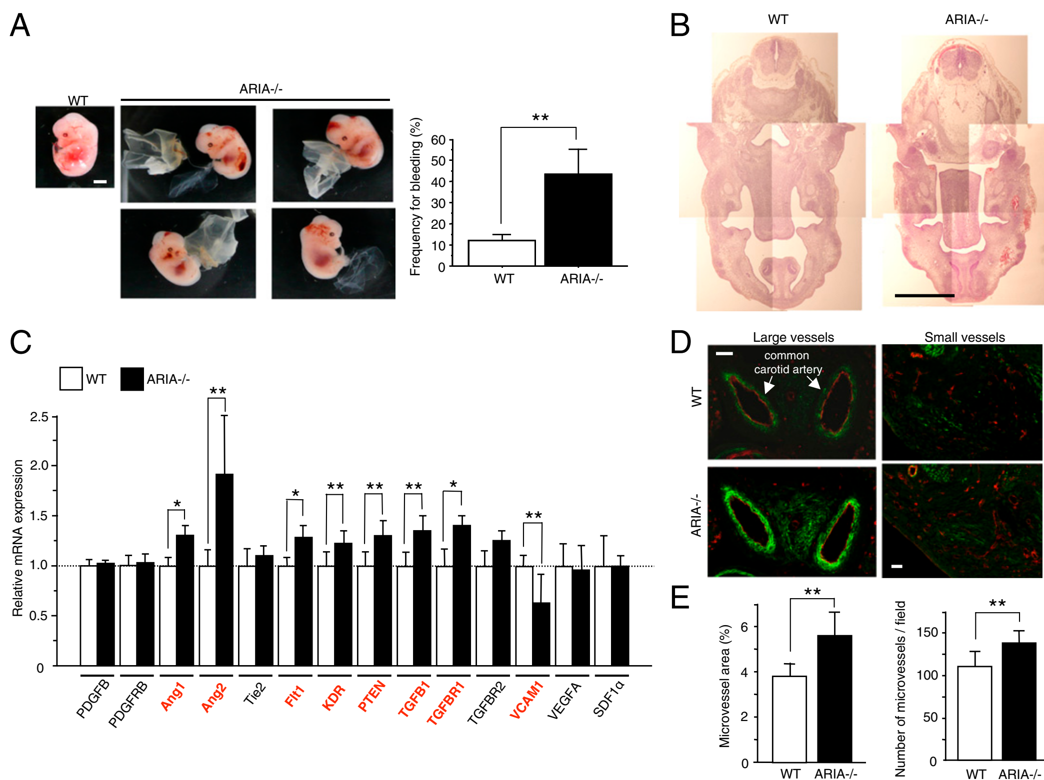

Fig. 2. ARIA is involved in the embryonic vascular development. (*A*) Representative pictures for wild-type (WT) or ARIA$^{-/-}$ mouse embryos at e14.5d. The frequency of bleeding observed in WT ($n = 57$) or ARIA$^{-/-}$ ($n = 51$) mouse embryos at e14.5d was shown. (*B*) Sections of WT or ARIA$^{-/-}$ mouse embryos at e14.5d were stained with hematoxylin and eosin. (*C*) Quantitative real-time PCR of the vascular growth factors expressed in whole yolk sacs extracted from WT or ARIA$^{-/-}$ embryos at e14.5d ($n = 8$ each). (*D*) Sections of WT or ARIA$^{-/-}$ mouse embryos at e14.5d stained with isolectin-IB4 (red) and anti-αSMA antibody (green). (*E*) Quantitative analysis of small vessel density for WT ($n = 6$) or ARIA$^{-/-}$ ($n = 8$) mouse embryos. Data are represented as mean ± SEM. *$P < 0.01$, **$P < 0.05$. [Scale bars, 1.0 mm (*A* and *B*); 30 μm (*D*).]

Fig. 3. Postnatal neovascularization is augmented in ARIA-knockout mouse. (*A*) Relative change in blood flow detected by laser Doppler flowmetry in limbs of WT or ARIA$^{-/-}$ mice with critical limb ischemia (n = 6 each). (*B*) Sections of ischemic adductor muscles of WT or ARIA$^{-/-}$ mice were stained with isolectin IB4 (n = 6 each). Capillary density was quantitatively analyzed. (Scale bar, 50 µm.) (*C*) Bone marrow (BM) cells were extracted from ARIA$^{+/+}$–GFP^{+} or ARIA$^{-/-}$–GFP^{+} mice and transplanted into WT or ARIA$^{-/-}$ mice. Blood flow detected by laser Doppler in ischemic limbs of BM-chimeric mice (n = 6 each) was shown. (*D*) Quantitative analysis of capillary density in ischemic adductor muscles (n = 6 each) of the BM-chimeric mice. (*E*) The number of BM-derived ECs in ischemic muscles was quantitatively analyzed (n = 6 each). Data are represented as mean ± SEM. *P < 0.00001, **P < 0.001, ***P < 0.05.

ARIA expression significantly increased, whereas ARIA silencing reduced, phospho-PTEN (Ser380/Thr382/Thr383) in the membrane fraction to the same extent as total PTEN (Fig. 5*C* and Fig. S6*B*). In support of these findings, ARIA associates with phosphomimic recombinant PTEN as well as wild-type and nonphosphomimic recombinant PTEN (Fig. 6*A*). Furthermore, ARIA associates with PTEN in which the C-terminal region including the PDZ-binding motif was deleted, indicating that the C-terminal region of PTEN is not required for the association between PTEN and ARIA (Fig. 6*A*).

Given that ARIA is a single-span type-I transmembrane protein, the C-terminal intracellular domain (ICD) of ARIA is

Fig. 4. ARIA regulates neovascularization by modulating the PI3K/Akt/eNOS signaling in vivo. (*A*) Akt/eNOS signaling in aortic ECs isolated from WT or ARIA$^{-/-}$ mice. Immunoblotting, in which two independently prepared samples were used, was repeated three times, and the representative results are shown. The quantitative data are shown in Fig. S5. (*B*) Migration toward 2% FBS, apoptosis (TUNEL-positive cells, red and nuclei, blue) and tube formation on Matrigel of aortic ECs isolated from WT or ARIA$^{-/-}$ mice. (Scale bar, 200 µm.) (*C*) Blood flow detected by laser Doppler in ischemic limbs of WT or ARIA$^{-/-}$ mice treated with vehicle, NOS inhibitor (L-NAME), or PI3K inhibitor (ZSTK474) (n = 6 each). (*D*) Quantitative analysis of capillary density in ischemic adductor muscles (n = 6 each). Data are represented as mean ± SEM. *P < 0.00001, **P < 0.05.

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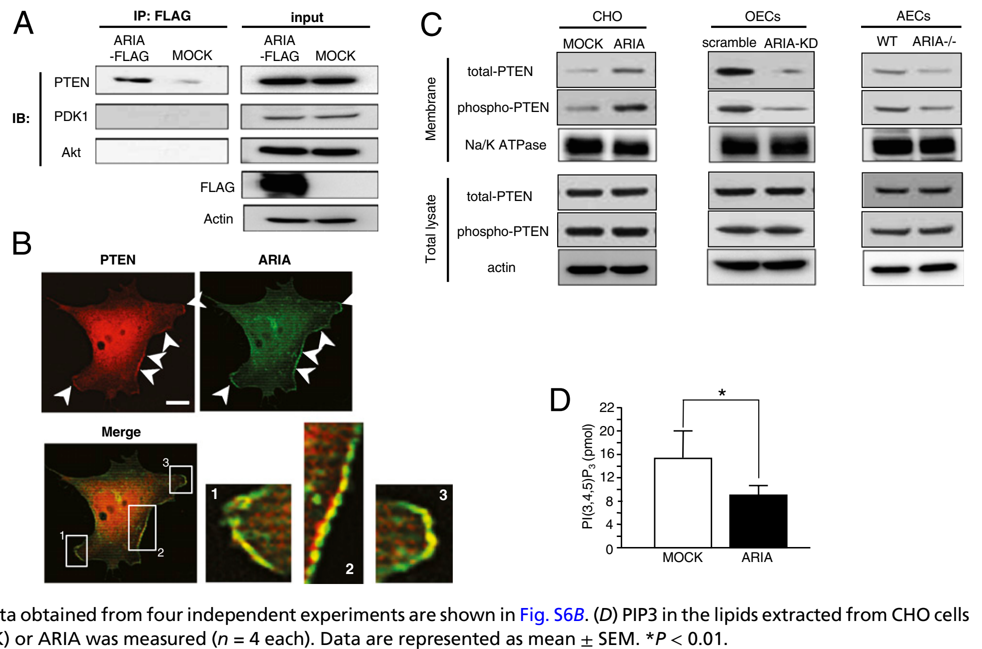

Fig. 5. ARIA interacts with PTEN and regulates the membrane-association of PTEN. (*A*) Recombinant ARIA–FLAG expressed in py4.1 cells was immunoprecipitated. Total cell lysates (input) or proteins coprecipitated with ARIA were detected with antibodies for target proteins. Results were confirmed by three independent experiments. (*B*) HA–PTEN and ARIA–FLAG expressed in py4.1 cells was detected with anti-HA (PTEN, red) or anti-FLAG (ARIA, green) antibodies. Enlarged images of the merged picture in white rectangular outlines are also shown. Similar results were observed in three independent experiments. (Scale bar, 10 μm.) (*C*) Total or phosphorylated PTEN (Ser380/Thr382/Thr383) was detected in the membrane fraction or total cell lysate extracted from: CHO cells stably transfected with empty vector (MOCK) or ARIA plasmid, OECs transfected with scramble or ARIA siRNA (ARIA KD), and mouse aortic ECs (AECs) isolated from WT or ARIA$^{-/-}$ mice. The representative results of immunoblotting are shown. Quantitative data obtained from four independent experiments are shown in Fig. S6*B*. (*D*) PIP3 in the lipids extracted from CHO cells stably transfected with empty vector (MOCK) or ARIA was measured (n = 4 each). Data are represented as mean ± SEM. **P* < 0.01.

considered to bind to PTEN. To confirm this, we prepared expression constructs for ARIA ICD in which the extracellular and membrane domain were deleted, and for ARIA extracellular domain (ECD) which do not have ICD. Recombinant ARIA ICD was localized in the cytosol and nucleus, and no significant accumulation on the plasma membrane was observed, whereas ARIA ECD showed significant membrane localization (Fig. S7*A*). PTEN was coprecipitated with ARIA ICD but not with ARIA ECD, which further suggests that ARIA associates with PTEN at its ICD on the membrane (Fig. S7*B*). Moreover, we detected their association in live cells using fusion proteins of complementary fragments of a coral fluorescent reporter protein (Fig. S7*C*). Of note, only a pair of fusion proteins of ARIA with the complementary fragments of a reporter protein at C-terminal and PTEN with the complementary fragments of a reporter protein at N-terminal showed the signals of the reconstituted fluorescence (Fig. S7*C*). Therefore, it appears that the N-terminal region of PTEN is important for the association between PTEN and ARIA. These collectively indicate that ARIA associates with and anchors PTEN to the membrane independently of the PTEN phosphorylation in the C-terminal tail and consequently inhibits the PI3K signals (Fig. 6*B*).

Discussion

In the present study, we identified a unique molecular basis for the regulation of the PTEN/PI3K pathway in ECs and EPCs by ARIA, which controls developmental and postnatal angiogenesis. The PI3K/Akt/eNOS axis constitutes a major determinant in postnatal angiogenesis at ischemic sites (14, 19). Also, the pivotal role of PTEN in ECs' angiogenic functions as well as postnatal neovascularization has been reported (18, 28). Akt controls various endothelial functions, such as apoptosis, migration, tube formation, and NO production (13, 14), whereas it is also known as an oncogene due to its potent antiapoptotic property. To avoid the out-of-control function of Akt, PI3K/Akt signals are closely regulated by endogenous inhibitors including PTEN.

PTEN needs to locate near the membrane to antagonize PI3K. Various modifications such as phosphorylation, ubiquitination, acetylation, and oxidation modulate PTEN function, and phosphorylation of the C-terminal tail plays a significant role in the

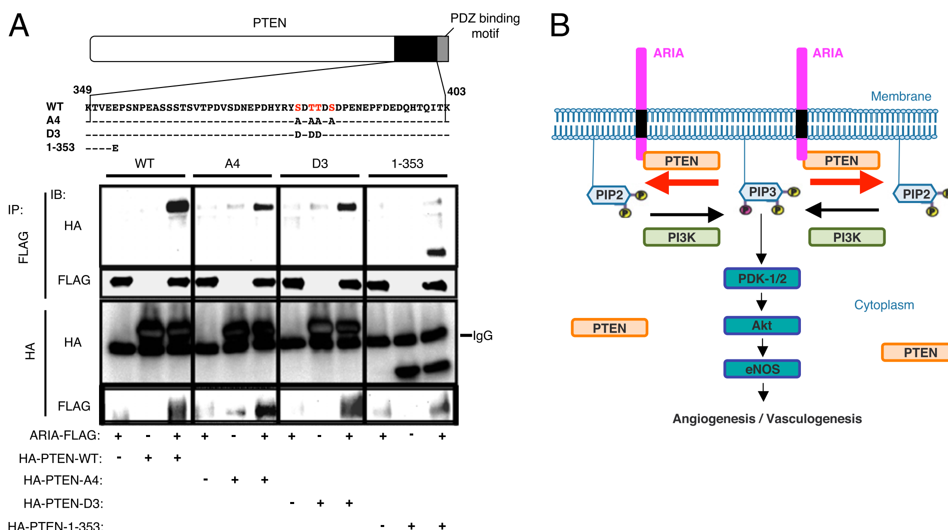

Fig. 6. ARIA is a unique factor in the regulation of the PTEN/PI3K pathway. (*A*) WT, nonphosphomimic (A4), phosphomimic (D3), or C-terminal tail-deleted (1-353) PTEN tagged with HA, and ARIA–FLAG were transfected into CHO cells. Coprecipitation of each mutant PTEN and ARIA was analyzed. Results were confirmed by three independent experiments. (*B*) Proposed model for ARIA function. ARIA associates with PTEN at its intracellular domain and anchors PTEN to the membrane. ARIA enhances the membrane localization of PTEN and consequently reduces the PI3K/Akt signaling in ECs and EPCs, leading to the negative regulation in angiogenesis and vasculogenesis.

regulation of its membrane association (23). In addition, candidate proteins that can recruit PTEN to the membrane have been previously identified, including the MAGI proteins, NHERF proteins, and neutral endopeptidase (28–32). The most striking difference between ARIA and these proteins is that the C-terminal region including the PDZ-binding motif of PTEN is not required for the binding of PTEN to ARIA, and phosphorylation of the C-terminal tail of PTEN is unlikely to affect the binding affinity of PTEN with ARIA. Thus, our work has revealed a previously unknown mode of regulation of PTEN activity.

Targeted deletion of PTEN leads to embryonic lethality at an early developmental stage of gestation (33). Recently, it has been reported that EC-specific PTEN deletion also causes embryonic lethality around embryonic day 11.5 due to bleeding and cardiac failure (18). Thus, PTEN plays an essential role in the cardiovascular development. We found that gene targeting of ARIA caused peripheral bleeding during embryogenesis without apparent developmental delay. In contrast to EC-specific PTEN$^{-/-}$ embryos, pericytes and/or vascular smooth muscle cell coverage for large vessels was not impaired in ARIA$^{-/-}$ embryos. Nevertheless, the altered expression pattern of vascular growth factors in yolk sacs of ARIA$^{-/-}$ embryos was similar to that in EC-specific PTEN$^{-/-}$ embryos (18). On the other hand, an increased small vessel density and bleeding detected in ARIA$^{-/-}$ embryos was similar to those observed in EC-specific Bcl-2 transgenic mice embryos (34), suggesting that reduced endothelial apoptosis might predominantly contribute to the inappropriate developmental angiogenesis in ARIA$^{-/-}$ embryos.

Our previous study demonstrated that ARIA knockdown reduced apoptosis, but did not affect migration in HUVECs (20). These results differ presumably due to insufficient silencing of ARIA in HUVECs in the previous setting (~60% reduction in mRNA) compared with that in OECs in the current experiments (~98% reduction in mRNA).

Our present data using gene targeting of ARIA in addition to gene knockdown by siRNA clearly and consistently showed that silencing of ARIA enhanced migration and tube formation of ECs and OECs and that ARIA negatively regulates ischemia-induced neovascularization. High and preferential expression of ARIA in ECs and EPCs/OECs gives rise to the possibility that regulating ARIA function could modulate neovessel formation through the modification of PTEN/PI3K/Akt pathway without inducing detrimental conditions such as oncogenicity. We observed no adverse effect of ARIA deletion in adult mice so far, suggesting the feasibility of ARIA inhibition as a therapeutic approach. The capacity of ARIA to regulate the neovascularization through a newly identified mechanism might offer a therapeutic advantage. Nevertheless, further analysis is required, especially exploring whether ARIA is also involved in other PTEN-controlled biological processes.

Materials and Methods

Cell Culture. Human early and late EPCs were isolated from peripheral blood as described previously (1). Briefly, mononuclear cells were isolated by centrifugation through a Histopaque-1077 (Sigma) density gradient and then cultured on human fibronectin-coated dishes (BD Bioscience) in EGM-2 medium (Clonetics). Early growth spindle-shaped EPCs emerged within 2 wk, whereas late outgrowth cobblestone-shaped EPCs appeared at 3–4 wk. The expression of KDR and the uptake of acetylated LDL were confirmed in both early and late EPCs. Late outgrowth EPCs isolated from cord blood were obtained from BioChain. Aortic ECs were isolated from descending thoracic aortas of wild-type and ARIA$^{-/-}$ mice. Under a dissecting microscope, 1 mm-thick aortic rings were prepared, and plated onto culture plates containing EGM-2 medium. EC sprouting was observed after 3–4 d, and the cells were subcultured once before experiments. The expression of CD31 was detected in more than 90% of cells.

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