Vascular RhoJ Is an Effective and Selective Target for Tumor Angiogenesis and Vascular Disruption

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SUMMARY

Current antiangiogenic therapy is limited by its cytostatic nature and systemic side effects. To address these limitations, we have unveiled the role of RhoJ, an endothelial-enriched Rho GTPase, during tumor progression. RhoJ blockade provides a double assault on tumor vessels by both inhibiting tumor angiogenesis and disrupting the preformed tumor vessels through the activation of the RhoA-ROCK (Rho kinase) signaling pathway in tumor endothelial cells, consequently resulting in a functional failure of tumor vasculatures. Moreover, enhanced anticancer effects were observed when RhoJ blockade was employed in concert with a cytotoxic chemotherapeutic agent, angiogenesis-inhibiting agent, or vascular-disrupting agent. These results identify RhoJ blockade as a selective and effective therapeutic strategy for targeting tumor vasculature with minimal side effects.

INTRODUCTION

Tumor angiogenesis is a prerequisite for tumor progression (Ferrara and Altimalo, 1999; Hanahan and Folkman, 1996). The angiogenic switch is activated during tumor growth, and the resulting tumor neovessels manage the O2 and nutrient requirements as well as the clearance of CO2 and metabolite in tumor tissue (Carmeliet and Jain, 2011; Hanahan and Folkman, 1996). Moreover, the tumor vasculature is one of the main routes of tumor cell metastasis to distant organs (Hanahan and Weinberg, 2011). Collectively, these observations imply that tumors cannot grow further and metastasize without sufficient blood supply. This inference has led to the development of various angiogenesis-inhibiting agents (AIAs) in the past decade (Ellis and Hicklin, 2008; Ferrara and Kerbel, 2005; Sennino and McDonald, 2012), many of which target vascular endothelial growth factor (VEGF) and its receptors and have proved to be effective in clinical practice (Carmeliet and Jain, 2011; Chung et al., 2010). In addition, ongoing drug development has focused on moderating other angiogenic pathways (Bono et al., 2013; Gerald et al., 2013; Koh et al., 2010; Sennino and McDonald, 2012; Tvorogov et al., 2010). Because current AIAs are inherently cytostatic and target newly growing tumor vasculature, they are more suited to tumor stabilization than to the regression of a bulky tumor (Ellis and Hicklin, 2008; Horsman and Siemann, 2006). Even after repeated cycles of AIA treatment, a substantial amount of...
Results

High Expression of RhoJ in Tumor ECs during Tumor Progression

To unveil the role of RhoJ in tumor progression, we generated RhoJ-KO (RhoJ-KO) mice, in which RhoJ is knocked out by replacing its exon 1 with GFP; with this construct, GFP is expressed instead of RhoJ under the transcriptional control of the RhoJ promoter (Figures S1A–S1C available online). To monitor RhoJ expression in tumor tissues, RhoJ-KO mice were implanted with Lewis lung carcinoma (LLC) and B16F10 melanoma cells. To observe RhoJ expression in a spontaneous breast tumor model, we generated MMTV-PyMT/RhojGFP/+ mice (P/Rhoj GPP/+ ) by mating RhojGFP/+ mice with MMTV-PyMT/+ mice. The LLC tumor and B16F10 melanoma displayed high RhoJ expression in tumor vessels 7 days after implantation, and spontaneous breast tumors of P/RhojGFP/+ also showed strong RhoJ expression in tumor vessels 12 weeks after birth (Figure 1A). In contrast to robust expression in tumor vessels, RhoJ expression was not observed in the lymphatic vessels of tumors and lymph nodes (LNs). High-magnification analyses of the LLC tumor revealed that RhoJ expression was mainly confined to tumor ECs, whereas some non-ECs such as perivascular mural cells and tumor stromal cells also occasionally expressed RhoJ (Figure 1B). Quantitative RT-PCR analysis of purified cells from LLC tumors showed that they consistently exhibited a predominant expression of RhoJ in CD31+CD45− tumor ECs with a weak expression in CD31−CD45− cells, but no expression in CD31+CD45+ hematopoietic cells (Figure 1C). To identify RhoJ-expressing stromal cells other than ECs, GFP+ and GFP− cells were purified from the CD31−CD45+ cells of RhoJ−/−/− tumor vessels using fluorescence-activated cell sorting (Figure S1D). We discovered that these RhoJ-expressing non-ECs highly expressed platelet-derived growth factor (PDGF)Rα, PDGFβ, α-SMA, and FSP-1, indicating that these cells could be pericytes and cancer-associated fibroblasts (Figure S1E). In tumor vasculature, RhoJ expression follows a distinct spatio-temporal regulation. It is most robustly expressed during early tumorigenesis, in contrast to being attenuated in later stages of tumor growth (Figures 1C–1E). Moreover, RhoJ is intensively expressed in the peritumoral high-angiogenic region compared to the intratumoral regions of various tumors (Figures 1F, 1G, and S1F). Intriguingly, RhoJ expression in normal tissues of adult mice is very infrequent and indistinct, only occasionally present in heart blood vessels and stromal cells and in LH blood vessels (Figure S1G). RhoJ-KO mice grew to adulthood normally without any growth retardation or vascular abnormalities in major organs including heart, lung, kidney, and liver (Figure S1H). Also, there were no differences in vascular morphology and integrity between RhoJ-KO mice and wild-type (WT) mice (Figures S1I–S1O). These findings suggest that RhoJ is a potential candidate for a more selective vascular targeting therapy with attenuated systemic side effects compared to current AIA therapies.

To examine the relevance of RhoJ in human tumor angiogenesis, we assessed RhoJ expression in human tissues and confirmed that RhoJ is highly expressed in the tumor vessels of colon adenocarcinomas (7 of 12 samples), but it is undetectable in normal colon tissues (0 of 10 samples) (Figure 1H). Furthermore, we analyzed the RhoJ expression using the 216 colon cancer
patients data set of The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov) (Table S1) and found that the patients that had tumors with high RhoJ expression had increased prevalence of lymphovascular invasion (Figure 1I) and decreased overall survival after the diagnosis of colon cancer (Figure 1J). Finally, the RhoJ expression positively correlated with the number of metastatic LNs (Figure 1K), suggesting the possible positive correlation of RhoJ with human cancer progression.

RhoJ Deletion Suppresses Tumor Growth, Neoangiogenesis, and Metastasis in the LLC Tumor

Taking the advantage that RhoJ-KO mice grew to adulthood normally, we used RhoJ-KO mice to address the role of RhoJ during tumor progression. We employed the LLC tumor model by subcutaneously (s.c.) injecting LLC cells into RhoJ-WT and KO mice. At 3 weeks after implantation, compared to WT mice, RhoJ-KO mice showed a 55% reduction in tumor growth.
Targeting RhoJ for Tumor Vascular Disruption

RhoJ Deletion Disrupts Tumor Vascular Integrity and Function

Tumor vasculature consists of malformed, disintegrated, leaky, and highly branched vessels that continuously undergo vascular remodeling (McDonald and Baluk, 2002; Siemann, 2011; Trédan et al., 2007). Because RhoJ-KO mice displayed increased intratumoral hemorrhage compared to RhoJ-WT mice, we further investigated the role of RhoJ in vascular integrity and function. Interestingly, the LLC tumor of RhoJ-KO mice had more disrupted tumor vessels (Figure 3A) and reduced vascular density (Figure 2M and 2N). The number of metastatic tumor colonies (>100 μm in diameter in tumor sections) in the lungs was 51% less in RhoJ-KO mice (Figures 2M and 2N).

Consistent with the findings observed in LLC tumors, tumor growth was delayed by 52% in RhoJ-KO mice compared to RhoJ-WT mice (Figures S3A and S3B). In terms of tumor angiogenesis, vascular densities were reduced by 31% and 28% in the peri- and intratumoral regions of RhoJ-KO mice, respectively (Figures S3C and S3D). Moreover, lymphatic metastasis of tumor cells into inguinal LNs was suppressed by 47% in RhoJ-KO mice (Figures S3E and S3F).

RhoJ Deletion Also Reduces Tumor Growth, Neovessel Formation, and Metastasis in the Spontaneous Breast Cancer Model

As for the spontaneous tumor model, MMTV-PyMT mice were treated with RhoGFP/GFP to generate MMTV-PyMT;RhoJ+/+ mice (P/RhoJ-WT) and MMTV-PyMT;RhoGFP/GFP mice (P/RhoJ-KO). At 14 weeks of age, P/RhoJ-KO showed reduced development of spontaneous mammary tumor nodules compared to P/RhoJ-WT (Figure 4A). In the P/RhoJ-KO, compared to those in P/RhoJ-WT, median time to palpable tumor development was delayed by ∼2 weeks (Figure 4B), and the number of tumor nodules per mouse decreased 32%–41% (Figure 4C). Moreover, the average size and tumor burden of P/RhoJ-KO mice were 61% and 64% less, respectively, than those of P/RhoJ-WT mice (Figures 4D and 4E). Histological examination showed that there were more noninvasive carcinoma lesions with well-preserved tumor margins in the peritumoral regions of P/RhoJ-KO (Figure 4F, see legend for a detailed explanation), which indicates that RhoJ deficiency delays tumor progression and invasion. Also, in P/RhoJ-KO, vascular densities were 35% and 41% less (Figures 4G and 4H) and tumor vascular sprouting was 59% and 42% less (Figure 4I) in the peri- and intratumoral regions, respectively, compared to P/RhoJ-WT. Furthermore, morphology of tumor vasculatures in the intratumoral regions of P/RhoJ-KO seemed more disrupted (Figure 4G). Consistent with their disintegrated morphology, extravasation of i.v.-injected dextran was 2.5-fold greater in the tumor vessels of P/RhoJ-KO (Figures 4J and 4K), indicating that P/RhoJ-KO tumor vessels are highly permeable compared to those of P/RhoJ-WT. In addition, PDGFRβ+ pericyte support was 62% less (Figures 4L and 4M) and the track of collagen type IV+ BM along tumor vessels was 46% less (Figures 4N and 4O) in P/RhoJ-KO. Finally, the number of metastatic tumor colonies (>100 μm in diameter in tumor sections) in the lung was 82% less in P/RhoJ-KO (Figures 4P and 4Q). Taken together, these results lead us to conclude that RhoJ plays a crucial role in the formation of tumor neovessels and maintenance of tumor vascular integrity, influencing the tumor progression.

RhoJ Deficiency Delays Wound Healing through Attenuated Angiogenesis

We also evaluated the role of RhoJ in wound healing using a punch-wound healing model. Like tumor vessels, the blood vessels in the granulation area of wounds displayed high RhoJ expression (Figure S4A). Compared to RhoJ-WT mice, RhoJ-KO mice showed 23% delayed wound closure, 48% reduced vascular density, and 39% decreased granulation area in the wound regions (Figures S4B–S4G). Thus, RhoJ plays a positive angiogenic role in wound healing.
Figure 2. RhoJ Deletion Inhibits Tumor Growth, Neovessel Formation, and Metastasis in LLC Tumors

Three weeks after implantation of LLC cells into RhoJ-WT and -KO mice, histological analyses were performed. Unless otherwise denoted: scale bars, 100 μm. Each group: n = 6. Values are mean ± SD. *p < 0.05 versus RhoJ-WT.

(A and B) Comparisons of tumor volume (A) and growth rate (B). Each group: n = 10.

(C) Tumor sections stained with H&E. Arrows indicate hemorrhagic lesions. Scale bar, 5 mm.

(D) Intratumoral bleeding area (%). WT KO

(E) Hypoxprobe CD31

(F) CD31 Caspase3

(G) Peritumoral intratumoral

(H) Peritumoral BV density (%)

(I) WT KO

(J) Sprout No./mm²

(K) LN metastasis area (%)

(L) WT KO

(M) RhoJ-WT RhoJ-KO

(N) Metastatic colony/section

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Targeted RhoJ Deletion in Tumor ECs Suppresses Tumor Angiogenesis and Disrupts Tumor Vessel Integrity

To ascertain the role of RhoJ in tumor ECs during tumor angiogenesis, we generated inducible EC-specific RhoJ loss-of-function mice (Rhoj-WT) by mating Rhoj-WT with Cdh5(PAC)-CreERT² (Wang et al., 2010), in which the Rhoj allele was efficiently deleted in the ECs upon tamoxifen administration (Figures S5A–S5C). Rhoj-WT mice (Rhoj-WTEC) were used as control. Compared to those in Rhoj-WTEC, LLC tumors showed 38% reduced growth in Rhoj-KOEC (Figure 5A) and remarkable intratumoral hemorrhagic necrosis 16 days after LLC tumor implantation (Figure 5B), in which the hemorrhagic area was 3-fold larger and viable tumor areas were 35% less (Figures 5C and 5D). The impact of EC-specific Rhoj deletion on tumor growth was 31% less compared to global Rhoj deletion, which is attributed by the 70% deletion of Rhoj in tumor ECs (Figure S5C). Vascular densities of Rhoj-KOEC tumors were 45% and 43% reduced in the peri- and intratumoral areas, respectively (Figures 5E and 5F). Notably, the morphology of tumor vessels in the intratumoral core of Rhoj-KOEC was more disrupted (Figure S5D). Additionally, PDGFRα+ pericyte coverage was reduced by 68% (Figures 5G and 5H), and collagen type IV+ BM coverage along tumor vessels was 51% diminished (Figures 5I and 5J) in Rhoj-KOEC. Moreover, the leakage of i.v.-injected dextran was remarkably increased by 6.8-fold in the intratumoral core of Rhoj-KOEC (Figures 5K and 5L). Finally, junctional CD144 expression seemed to be decreased in the intratumoral regions of Rhoj-KOEC (Figure S5E). We also evaluated the effect of endothelial Rhoj deletion on established macroscopic tumors (>300 mm³). The results showed that the tumor growth was decreased by 34% (Figure 5M) and the overall survival of mice increased by ~25% (Figure 5N), denoting that Rhoj is a feasible target for further anticancer drug development even in established tumors. In conclusion, these findings indicate that Rhoj in tumor ECs is critical to the regulation of tumor angiogenesis and maintenance of tumor vascular integrity.

RhoJ Regulates EC Motility, Tube Formation, and Junctional Integrity through Suppression of the Rhoa-ROCK Signaling Pathway

To determine the role of Rhoj in in vitro angiogenesis and vascular leakage, HUVECs transfected with either Rhoj siRNA (si-J-ECs) or control siRNA (si-C-ECs) were used. To exclude the off-target effects, five independent Rhoj siRNA were designed, and three Rhoj siRNAs with the best performance (named J0, J1, and J2) were chosen for further experiments (Figures S6A and S6B), and their results were averaged (si-J-EC) (see Figure S6 for their individual results). In comparison to those of si-C-ECs, a time-lapse tracking analysis revealed that si-J-ECs displayed more restricted motility with a 54% reduction in displacement speed and 27% reduction in trajectory speed (Figures 6A, 6B, and S6C). Moreover, our established microfluidics assay (Joo et al., 2012) (Figures S6D and S6E) showed that si-J-ECs had 38% and 50% less migration and angiogenic sprouting, respectively (Figures 6C and 6D), indicating that Rhoj is an important regulator of EC migration and sprouting. Furthermore, si-J-ECs on the Matrigel formed poorly connected networks with decreased numbers of EC junctions and tubules (Figures 6E, 6F, and S6F). In addition, detailed analysis of ECs and EC tubules showed that si-J-ECs have increased actin stress fiber formation (Figures 6G and S6G). In agreement with this result, increased actin stress fiber formation was also observable in the intratumoral vasculatures of Rhoj-KOEC (Figure 6H), confirming the negative correlation between Rhoj and EC stress fiber formation (Kaur et al., 2011).

We next questioned whether Rhoj has any role in maintaining EC integrity, because various Rho GTPases are also involved in endothelial integrity (Beckers et al., 2010; Bryan and D’Amore, 2007). To answer this question, an in vitro vascular permeability assay was applied to examine the changes in EC paracellular integrity (Figure 6I). Compared to si-C-ECs, the vascular permeability across the EC monolayer was increased by 55% and 134% with or without VEGF-A, respectively, in si-J-ECs (Figures 6J and S6H). Consistent with this finding, junctions between ECs were also more loose and disrupted in si-J-ECs, especially in the presence of VEGF-A (Figures 6K and 6L). In parallel, tumor blood vessels of Rhoj-KO mice were also seriously disrupted (Figure S6I). These suggest that Rhoj works to maintain the integrity of the EC monolayer and negatively regulates VEGF-A-induced vascular leakage. Finally, we questioned whether Rhoj is associated with Rhoa-ROCK-myosin signaling, because this signaling is an important regulator of stress fiber formation and EC contraction (Sun et al., 2006) and endothelial Rhoj also seemed to be related to the regulation of stress fibers. Indeed, si-J-ECs had increased Rhoa activity, ROCK activity, and myosin light-chain phosphorylation, but these almost completely diminished with the ROCK inhibitor, Y-27632 (Figures 6M and S6J–M), suggesting that Rhoj is a negative regulator of the Rhoa-ROCK signaling pathway in ECs. Collectively, these findings indicate that Rhoj plays an important role in EC migration, tube formation, and maintenance of vascular integrity through the suppression of the Rhoa-ROCK signaling pathway in ECs (Figure 6N).

(C) Comparison of intratumoral hemorrhagic area. Each group: n = 10.
(D) Comparison of intratumoral hemorrhagic area. Each group: n = 10.
(E) Images showing CD31+ blood vessels, caspase-3+ apoptotic cells, and Hypoxyprobe-1+ hypoxic areas in a tumor. Hypoxyprobe-1 was i.p.-injected 90 min before tumor sampling.
(F) Images showing CD31+ blood vessels, caspase-3+ apoptotic cells, and Hypoxyprobe-1+ hypoxic areas in a tumor. Hypoxyprobe-1 was i.p.-injected 90 min before tumor sampling.
(G and H) Images (G) and quantification (H) of blood vessels in the peri- and intratumoral regions.
(I and J) Images (I) and quantification (J) of vascular sprouts (arrows, sprout > 10 μm in length) of tumor vessels. Scale bars, 10 μm.
(K and L) Images (K) and quantification (L) of cytokeratin+ tumor metastasis in the inguinal LNs. The cytokeratin+ area was presented as a percent per total sectional area. Scale bar, 500 μm.
(M) Lung sections stained with H&E. Four regions were viewed under high magnification. Arrows indicate metastatic foci. Scale bar, 5 mm (upper) and 200 μm (lower).
(N) Comparison of number of metastatic colonies (>100 μm in diameter) per lung section. See also Figure S2.
Cisplatin Profoundly Retards Tumor Growth in RhoJ-Deleted Mice

To confirm the effect of RhoJ deletion in concert with conventional chemotherapeutic drugs, cisplatin (10 mg/kg) was intraperitoneally (i.p.) injected into RhoJ-KO mice once every week starting when tumor volume exceeded 100 mm³. Cisplatin significantly delayed LLC tumor growth by 90% in RhoJ-KO mice compared to a 64% decrease in RhoJ-WT mice (Figure S7A). Histological analyses after cisplatin treatment revealed 80% increased intratumoral necrosis in RhoJ-KO mice treated with cisplatin compared to RhoJ-WT mice treated with cisplatin (Figures S7B and S7C). In fact, intratumoral accumulation of cisplatin increased by ~2-fold in RhoJ-KO mice compared to RhoJ-WT mice (Figures S7D and S7E). This may be due to increased extravasation and retention of cisplatin from the disintegrated tumor vessels at the intratumoral region of RhoJ-KO mice. These findings indicate that RhoJ blockade in combination with conventional chemotherapy could yield an enhanced antitumor effect.

Dual Blockade of RhoJ and VEGF-A Signaling Displays a More Potent Antitumor Effect

Many Rho GTPases are activated by VEGF-A and share their common downstream effector molecules (Beckers et al., 2010; Beckers et al., 2010).
Schiller, 2006). Therefore, there is a possibility that VEGF-A-driven activation of other Rho GTPases may partially compensate for the effects of RhoJ ablation, limiting the antitumor effects of the RhoJ blockade. To resolve this potential problem and maximize the antitumor effect, we investigated the effect of VEGF-A blockade in the tumor progression of RhoJ-WT and KO mice. Administration of VEGF-trap (25 mg/kg) delayed LLC tumor growth by 88% in RhoJ-KO mice compared to a 47% decrease in RhoJ-WT mice (Figure 7A). Moreover, VEGF-trap reduced tumor vascular densities by 66% and 68% in peri- and intratumoral areas of RhoJ-KO mice, respectively, which was more potent than the 43% and 49% decrease in RhoJ-WT mice (Figures 7B and 7C). From these results, we could confirm the potential of RhoJ blockade as an adjuvant option to enhance AIA therapies, such as VEGF-trap.

Next, to establish a method for therapeutic blockade of RhoJ, a tumor-targeted siRNA delivery system (Kim et al., 2012) was employed. The aptide was designed and used according to a previous protocol (Figure S7F) (Kim et al., 2012). We chose fibronectin, an extracellular matrix (EDB) as the target for the aptide, because EDB is highly expressed in tumor tissues (Kim et al., 2012; Menrad and Menssen, 2005). The aptide specific for EDB (APT<sub>EDB</sub>) was conjugated with liposome to form an APT<sub>EDB</sub>-liposome complex, and siRNA was encapsulated within this APT<sub>EDB</sub>-liposome complex (Figure S7G). We confirmed the successful delivery of APT<sub>EDB</sub>-liposome into LLC tumor tissues (arrowhead, Figure S7H) and the knockdown of RhoJ with encapsulated RhoJ siRNA (en-siJ) compared to encapsulated control siRNA (en-siC) (Figures S7I–S7K). In vivo experiments showed that the antitumor effect of en-siJ (2 mg/kg) monotherapy or VEGF-trap (25 mg/kg) monotherapy was similar, showing a 40%–50% decrease in tumor volume compared to the control group, while the combination therapy of en-siJ with VEGF-trap increased this effect to 66% (Figure 7D). Moreover, en-siJ or VEGF-trap monotherapy decreased tumor vessel densities by ~45% and ~50% in the perivascular and intratumoral regions, respectively, but the combination therapy showed a 66% and 68% respective reduction (Figures 7E and 7F). Notably, intratumoral hemorrhage of en-siJ-treated tumors dramatically decreased with VEGF-trap treatment (Figures 7G and 7H), indicating that RhoJ blockade induces vascular disruption and hemorrhage in a VEGF-dependent manner, which is consistent with findings of the in vitro permeability assay (Figure 6J). Finally, the combination therapy showed a 75% reduction in LN metastasis, which was greater than either en-siJ or VEGF-trap monotherapy (Figures 7I and 7J). Together, the dual blockade of RhoJ and VEGF signaling is superior to the single blockade in antitumor, antiangiogenic, and antimetastatic activity.

**RhoJ Blockade Augments the Antitumor Effect of a VDA, Combretastatin-A4-Phosphate**

VDAs are known to disrupt established tumor vessels by directly targeting the cytoskeletons of ECs (Siemann, 2011). Because RhoJ blockade is comparable to VDAs in inducing tumor vascular disruption, we speculated that RhoJ blockade might have an enhancing effect with VDAs, such as combretastatin-A4-phosphate (CA4P). The in vitro tube formation assay revealed that RhoJ knockdown in concert with CA4P (20 nM) treatment profoundly inhibited EC tube formation, inducing almost complete disruption, compared to single treatment with either CA4P or RhoJ siRNA (Figures 8A and 8B). Also, permeability across the EC monolayer of siJ-ECs treated with CA4P was increased by 2.9-fold compared to that of siC-ECs treated with PBS (Figure 8C). Moreover, RhoJ knockdown in combination with CA4P further activated the RhoA-ROCK signaling pathway (Figure S8). These suggest a possible collaboration between RhoJ blockade and VDA treatments through complementation and enhancement of the vascular-disrupting effect. To confirm this hypothesis, we evaluated the influence of CA4P on RhoJ-WT and KO mice. Treatment with CA4P (50 mg/kg) resulted in only an 18% reduction in tumor growth of WT mice, in which the response to CA4P was not maintained and tumors began to regrow. However, RhoJ-KO mice displayed a 79% additional inhibition in tumor growth when treated with CA4P, in which a durable response to CA4P was observed. (Figure 8D). Moreover, CA4P reduced vascular densities by 59% and 60% in the peri- and intratumoral regions of RhoJ-KO mice, respectively, which was more potent compared to the respective 13% and 31% reduction in RhoJ-WT mice (Figures 8E and 8F). Finally, CA4P displayed an efficient antimetastatic effect in RhoJ-KO mice, reducing metastasis by 67%, but no reduction in RhoJ-WT mice (Figures 8G and 8H). Taking these data together, we confirmed that RhoJ blockade is a valuable complementary therapy to overcome the limitations of current VDA therapy.

**DISCUSSION**

Here, we have demonstrated a critical role of RhoJ in the regulation of tumor angiogenesis and tumor vascular integrity. The phenotypic endpoints of RhoJ blockade are similar to those of the blockade of another small GTPase, R-Ras; genetic disruption of R-Ras also severely impairs EC barrier function, resulting in disturbed tumor vascular maturation (Sawada et al., 2012). Furthermore, it has recently been reported that RhoJ is strongly expressed in human cancers, being one of the top ten genes of the common angiogenesis signature (Masiero et al., 2013). This correlates well with our data, which show that high expression of RhoJ in colon cancer is a negative prognostic factor in these patients, further highlighting RhoJ as a clinically relevant therapeutic target in cancer.

RhoJ blockade displayed several advantages over current vascular targeting therapy, but the most superior advantage is its “double assault” on tumor vessels. Vascular targeting agents developed during the past decade are commonly classified as either AIAs or VDAs. AIAs mainly suppress the formation of tumor neovessels and induce tumor vessel normalization, whereas VDAs directly disrupt preformed tumor vessels and shut down blood flow, finally resulting in massive tumor necrosis and hemorrhage (Tozer et al., 2005). AIAs are particularly effective in the peritumoral regions of newly progressing tumors where new tumor vessels are robustly developing, whereas VDAs are most effective in the intratumoral regions of an established tumor where preformed immature vessels are abundant (Horsman and Siemann, 2006; Siemann, 2011). RhoJ blockade encompasses the aspects of AIAs and VDAs and offers an effective strategy for targeting tumor vasculatures. It simultaneously impedes the formation of tumor neovessel and disrupts the pre-established tumor vessel network. Through this “double
Figure 4. RhoJ Deletion Delays Tumor Growth, Neovessel Formation, and Metastasis in the Spontaneous Breast Cancer Model

Tumor growth was analyzed weekly in spontaneous mammary tumors of P/RhoJ-WT and -KO starting from 8 weeks after birth. Samples were harvested 18 weeks after birth. Unless otherwise denoted: scale bars, 100 μm. Each group: n = 5. Values are mean ± SD. *p < 0.05 versus P/RhoJ-WT.

(A) Image showing tumor development at 14 weeks after birth. Dotted lines demarcate palpable mammary tumor nodules.

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assault” on tumor vasculature, RhoJ blockade markedly inhibited blood flow to tumor cells and displayed a convincing anti-cancer and antitemetastatic effect.

In addition, RhoJ blockade compensates for and augments other anti-cancer therapies. The combination therapy of RhoJ blockade and the conventional chemotherapeutic drug, cisplatin, proved to be very effective in delaying tumor progression. As is previously known, the intratumoral core of tumors is resistant to conventional anti-cancer therapies (Trédan et al., 2007; Wachsberger et al., 2003), because anticancer drug delivery to this core is limited and inefficient due to the immature tumor vessels and increased interstitial pressure (Fukumura and Jain, 2007). Additionally, tumor cells in the intratumoral core have an intrinsic resistance to chemotherapy because they proliferate slowly and the growth fraction is small (Trédan et al., 2007). Intriguingly, RhoJ blockade preferentially induces vascular shutdown in intratumoral regions, resulting in necrosis of the tumor cells. By combining cisplatin and the RhoJ blockade, both of which exert distinctive modes of action, we achieved a comparatively enhanced antitumor and antitemetastic effect, which suggests the potential of RhoJ blockade as an adjuvant for conventional chemotherapies. Moreover, the combination of RhoJ blockade with VDAs also showed an enhanced antitumor efficacy. Most VDAs target the tubulin cytoskeleton of tumor ECs directly and induce activation of RhoA-ROCK signaling in tumor ECs, resulting in the rapid and selective disruption of the preformed tumor vessels (Siemann, 2011). However, despite promising preclinical results, they failed to show efficacy in clinical trials (Baguley and McKeage, 2012).

The major drawback of VDAs is that they mainly target the intratumoral core, leaving the remaining peripheral viable rim to regrow and even acquire resistance to VDAs (Horsman and Siemens, 2006; Tozer et al., 2005). In contrast, RhoJ blockade in the present study exerted its antitumor effect through inhibition of neovessel formation in both the peri- and intratumoral regions and also enhanced shutdown of pre-existing tumor vessels in the intratumoral regions. Furthermore, we found that RhoJ blockade shares its action mechanism with VDAs, also activating the RhoA-ROCK signaling pathway. In this regard, it is logical to speculate that RhoJ blockade may be complementary to current VDA therapies. Indeed, we confirmed that RhoJ blockade could overcome the resistance acquired from VDA monotherapies, such as CA4P, with regard to tumor growth and progression.

Previous studies have found that VEGF-A stimulation regulates the activity of various Rho GTPases, such as Cdc42, Rac1, and RhoA, whereas interactions among various Rho GTPases are poorly understood (Beckers et al., 2010; Bryan and D’Amore, 2007; Schiller, 2006). Blocking the RhoJ pathway over a prolonged period raises the possibility of compensatory activation of other Rho GTPases in tumor vessels, especially by Cdc42 and Rac1, which share common downstream effector molecules with RhoJ (Leszczynska et al., 2011). From this perspective, the concurrent inhibition of RhoJ signaling and VEGF-A signaling could be an attractive therapeutic strategy not only by enhancing current AIA therapy, but also by maximizing the vascular-disrupting effect of the RhoJ blockade. Indeed, our findings strongly support this possibility. The combination of RhoJ blockade and VEGF decoy receptor, VEGF-trap, showed comparatively potent antiangiogenic activity in both peri- and intratumoral areas of the LLC tumor, which is known to be resistant to conventional AIA therapies (Shojaei et al., 2007). Another possible benefit from this combination is that RhoJ blockade may maintain and maximize responses to the AIA therapies. It is known that tumor vessels regrow alongside the ghost tracks of remnant BM after cessation or during the resting period of AIA treatment (Mancuso et al., 2006). Intriguingly, we observed a severe loss of BM in RhoJ-deficient tumor vessels, indicating that concurrent RhoJ blockade might abolish remnant BM in concert with AIA and prevent tumor vessel from regrowth, finally resulting in a sustained response to the AIA therapies.

An additional advantage of RhoJ blockade is that it selectively targets tumor vessels with minimal systemic side effects. Current AIAAs influence normal vessels as well, because their main targets, VEGF-A and its receptors, are expressed ubiquitously. Therefore, they induce systemic side effects such as hemorrhage, hypertension, proteinuria, and delayed wound healing (Chen and Cleck, 2009; Kamba and McDonald, 2007). On the other hand, RhoJ expression is very specific to pathologic cancer and antimetastatic effect.

See also Figure S4.
conditions, especially in tumor tissues, while being rarely expressed in organs under normal physiologic conditions; the global deletion of RhoJ does not induce gross abnormalities and lethality. However, our results indicate that RhoJ plays a positive angiogenic role during wound healing, and this could be an unavoidable side effect of a putative RhoJ inhibitor.

Finally, RhoJ is a feasible target for clinical drug development. We could therapeutically target RhoJ in tumor tissues through an in vivo siRNA delivery system. Using the APTEDS-LS complex as a carrier, which has high specificity against tumor tissues (Kim et al., 2012), we effectively delivered siRhoJ into tumor tissues and significantly delayed tumor growth and metastasis, especially in concert with VEGF-trap. Consequently, we established a way to clinically inhibit RhoJ.

In conclusion, our evidence shows that RhoJ is a promising selective target in the tumor vasculature that governs the angiogenic process.

**Figure 5. EC-Specific Ablation of RhoJ Suppresses Tumor Angiogenesis and Induces Vascular Disruption**

(A–L) Histological and functional analyses were performed 16 days after implantation of LLC cells into RhoJ-WT EC and -KO EC. Mice were treated with i.p. injections of tamoxifen (4 mg/kg) four times every 2 days starting from the day before tumor implantation. Unless otherwise denoted: scale bars, 100 μm. Each group: n = 5. Values are mean ± SD. *p < 0.05 versus RhoJ-WT EC.

(A) Comparisons of LLC tumor growth.

(B) Images of tumor sections stained with H&E. Dotted lines demarcate intratumoral hemorrhagic area. Scale bar, 5 mm.

(C) Comparison of intratumoral hemorrhage area.

(D) Comparison of viable area in cross sections.

(E and F) Images (E) and quantification (F) of CD31+ blood vessels in the peri- and intratumoral regions. Dotted lines indicate boundaries between the skin and tumor. (G and H) Images (G) and quantification (H) of coverage of PDGFRα+ mural cells along CD31+ tumor vessels. Coverage of PDGFRα is presented as a percent of length that lies along CD31+ vessels.

(I and J) Images (I) and quantification (J) of loss of collagen type IV+ BM along CD31+ tumor vessels. Coverage of collagen type IV is presented as a percent of length that lies along CD31+ vessels.

(K and L) Images (K) and quantification (L) of dextran leakage area (green) from tumor vessels. Dextran+ area is presented as a percent per total sectional area.

(M and N) RhoJ-WT EC and -KO EC were treated with i.p. injections of tamoxifen (4 mg/kg) four times on the indicated days (arrows), after the tumor volume had exceeded 300 mm3. Each group: n = 10.

(M) Comparison of LLC tumor growth. *p < 0.05 versus RhoJ-WT EC.

(N) Comparison of overall survival after tamoxifen injection. p = 0.016 by log rank.

See also Figure S5.
processes of tumor angiogenesis and vascular integrity. The distinguishing characteristics of RhoJ blockade provide a strategy for overcoming the limitations of current vascular targeting therapies in patients with advanced cancer. Further development of specific RhoJ inhibitors is needed to ascertain their efficacy and safety in clinical settings.

Figure 6. RhoJ Regulates EC Motility, Tube Formation, and Integrity through Suppression of the RhoA-ROCK Signaling Pathway
HUVECs were transfected with either control siRNA (siC-ECs) or RhoJ siRNA (siJ-ECs). Unless otherwise denoted: scale bars, 100 μm. Each group: n = 5. Values are mean ± SD. *p < 0.05 versus siC-ECs.

(A and B) Random migration of ECs was tracked with time-lapse microscopy for 6 hr. (A) Trajectory images showing locomotion of individual ECs. (B) Comparisons of EC migratory speed.

(C and D) siC-ECs and siJ-ECs were seeded into the 3D microfluidics system, in which ECs migrate and sprout along growth factor gradient for 3 days. (C) Images showing directional migration and sprouting of ECs. Solid line, starting point; dotted line, point of maximal migration. (D) Comparisons of maximal distance of EC migration and EC sprouting (>10 μm in length).

(E–G) siC-ECs and siJ-ECs were seeded on Matrigel and incubated for 12 hr. (E) Images showing EC tube formation. (F) Comparisons of number of EC junctions and tubules. (G) Images showing F-actin fibers (red) in EC tubules. Arrows indicate collapse of ECs and increased actin stress fiber. Indicated region (square) is magnified in the right panel.

(H) Images showing F-actin fiber in LLC tumor 16 days after tumor implantation into RhoJ-WT EC and -KO EC. Arrows indicate increased actin stress fiber in tumor vessels. Indicated region (square) is magnified in the right panel.

(I and J) siC-ECs or siJ-ECs were cultured on cell inserts until an EC monolayer formed. Subsequently, the amount of dextran permeated across the monolayer with or without VEGF-A (50 ng/ml) was measured. (I) Schematic diagram showing in vitro permeability assay. (J) Comparison of vascular permeability across EC monolayer. *p < 0.05 versus siC-EC+PBS.

(K and L) siC-ECs or siJ-ECs were cultured on culture plates until an EC monolayer formed. Consecutively, the ECs were incubated with or without VEGF-A (50 ng/ml) for 1 hr. (K) CD144 junctions of EC monolayer in various conditions. (L) Electron microscopic images of EC monolayer in various conditions. Arrows indicate spatial gaps between adjacent ECs. Scale bars, 5 μm.

(M) Immunoblotting showing modulation of RhoA-ROCK signaling pathway by RhoJ. siC-ECs or siJ-ECs were cultured for 24 hr, and treated with or without Y-27632 (20 μM) for 1 hr. Three independent experiments show similar results.

(N) Schematic diagram showing the role of endothelial RhoJ. When RhoJ is activated in ECs, RhoJ suppresses RhoA-ROCK signaling, while activating N-WASP and PAK, which reorganize the cortical actin filaments in ECs. Upon RhoJ knockdown, RhoA-ROCK signaling is no longer suppressed in ECs, therefore inducing EC contraction through increased formation of actin stress fibers, eventually causing vascular shutdown. N-WASP, Neural Wiskott-Aldrich syndrome protein; PAK, p21-activated kinase.

See also Figure S6.
Figure 7. Dual Blockade of RhoJ and VEGF Signaling Suppresses Tumor Progression and Metastasis

(A–C) LLC implanted RhoJ-WT or -KO mice were given injections of VEGF-trap (VT) or Fc on the indicated days (arrows). Tumors were sampled 9 days after the first treatment. Scale bars, 100 μm. Each group: n = 5. Values are mean ± SD. *p < 0.05 versus WT, Fc; #p < 0.05 versus WT, VT.

(A) Comparison of tumor growth.

B

C

WT+Fc  WT+VT  KO+Fc  KO+VT
Intratumoral Peritumoral

Intratumoral

Peritumoral

D

E

WT  KO

WT  KO

F

WT  KO

WT  KO

G

en-siC  en-siJ

en-siC  en-siJ

H

en-siC  en-siJ

en-siC  en-siJ

I

J

LYVE-1  Cytokeratin

Cancer Cell

Targeting RhoJ for Tumor Vascular Disruption


(legend continued on next page)
**EXPERIMENTAL PROCEDURES**

**Mice**

Animal care and experimental procedures were performed under the approval (KA2011-17) of the Animal Care Committee of the Korea Advanced Institute of Science and Technology. Specific pathogen-free (SPF) C57BL/6J and MMTV-PyMT transgenic mice (FVB/N) were purchased from Jackson Laboratory. Rhoj<sup>GRFP/KO</sup> and Rhoj<sup>GRFP/KO</sup> mice and Cdh5(PAC)-CreER<sup>T2</sup> mice (Wang et al., 2010) were transferred and bred in our SPF facilities. To deplete Rhoj in MMTV-PyMT tumors, Rhoj<sup>GRFP/KO</sup> male mice were intercrossed with MMTV-PyMT male mice. To deplete Rhoj specifically in ECs, Rhoj<sup>GRFP/KO</sup> mice were intercrossed with Cdh5(PAC)-CreER<sup>T2</sup> mice. All mice were fed with ad libitum access to...
Cisplatin (10 mg/kg every 7 days, Sigma-Aldrich) was i.p. injected for cytotoxic encapsulated into APT EDB-LS complexes, were i.v. injected into tumor-in vivo, control or RhoJ siRNA (2 mg/kg, indicated schedule), which were 2 days, Sigma-Aldrich) was i.p. injected as a VDA therapy. As a control, equal lyses. Tamoxifen (4 mg/kg four times every 2 days, Sigma-Aldrich) was i.p. injected into Cdh5(PAC)-CreERT2,Rhojfl/fl mice starting from the day before tumor implantation or after the tumor volume had exceeded 300 mm³. Cisplatin (10 mg/kg every 7 days, Sigma-Aldrich) was i.p. injected for cytotoxic chemotherapy when tumor volume exceeded 100 mm³. VEGF-trap (25 mg/kg, indicated schedule) was s.c. injected as an AIa therapy: CA4P (50 mg/kg every 2 days, Sigma-Aldrich) was i.p. injected as a VDA therapy. As a control, equal amounts of Fo or PBS were injected in the same manner. To knock down RhoJ in vivo, control or RhoJ siRNA (2 mg/kg, indicated schedule), which were encapsulated into APTEDB-LS complexes, were i.v. injected into tumor-bearing mice.

**Human Tumor Specimens**

All human samples were collected by the tissue bank of Severance Hospital, Seoul, Korea, with the informed consents from the donors following the bioethics and safety regulations. All procedures regarding human samples were performed with the approval of the institutional review board (KH2013-02).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.12.010.

**AUTHOR CONTRIBUTIONS**

C.K., H.Y., and Y.F. designed and performed the experiments. Y.F. generated the RhoJ-KO mouse. C.K., H.Y., and Y.F. analyzed the data and wrote the manuscript.

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